

The Clinical and Genetic Epidemiology of Pediatric Idiopathic Epilepsy in Newfoundland and Labrador

by

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Abstract

Background: Epilepsy is a common and very heterogeneous neurologic disorder which shows a predilection towards the pediatric population. Idiopathic epilepsy (IE) refers to cases with no known underlying etiology other than a presumed genetic predisposition. IE accounts for approximately half of all cases of epilepsy. Newfoundland and Labrador has a tertiary referral center for pediatric neurology patients where virtually all children living on the Avalon Peninsula are treated. The current study aims to determine the clinical and genetic epidemiologic characteristics in this population.

Purpose: To describe the incidence, genetic and clinical epidemiology of pediatric IE in the province of Newfoundland and Labrador.

Methods: All children < 15 years of age with IE were ascertained through the provincial pediatric neurology clinic at the Janeway Child Health Centre. Family history, medical history and blood samples were obtained from affected children and their family members. All blood samples were screened for mutations in the seven candidate genes (*SCN1A*, *SCN2A*, *SCN1B*, *KCNQ2*, *GABRA1*, *GABRG2* and *KCNQ3*) of highest priority, based on previously reported phenotype-genotype relationships, by direct bidirectional Sanger DNA sequencing.

Results: The mean annual incidence of IE for the population of children living on the Avalon region of Newfoundland from 2000 to 2004 was 107 per 100 000. This rate is approximately three-fold greater than comparable populations in other developed countries. Incidence was similar in males and females. Multiple different epilepsy phenotypes were identified between

and amongst families. Of 117 families with IE eligible for study, 86 (74%) provided detailed pedigree data. Fifty-five families (64%) had a positive family history. Eight (9%) of these had family histories compatible with autosomal dominant (AD) inheritance and these families lived in five different geographic isolates. DNA was obtained from 79 individuals in 21 families. Seven genes previously associated with childhood epilepsy were sequenced and excluded in all but one family which had a pathogenic mutation in *SCN1A*. This gene encodes a sodium channel subunit and a novel missense mutation (c.1162T>C, p.Tyr388His) was identified in a multiplex family segregating a severe, clinically novel form of autosomal dominant generalized epilepsy with febrile seizures plus (GEFS+). The phenotype was consistent with GEFS+, with a variable phenotype that included mood and anxiety disorders, as well as ataxia – thereby expanding the phenotypic spectrum of GEFS+ to include neuropsychiatric disease.

Conclusions: The incidence of IE in the Avalon Peninsula of Newfoundland is three times higher than comparison populations and the rate of familial disease is high throughout the province of Newfoundland and Labrador. The distribution of familial and AD IE in different geographic isolates, together with the clinical heterogeneity of disease, suggests substantial genetic heterogeneity. A novel *SCN1A* mutation and phenotype was identified.

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I also wish to thank the family members for their willingness to participate in this study. Without the generous participation and cooperation of patients and family members, research projects such as this one would not be possible.

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List of Abbreviations and Symbols

AD:	Autosomal dominant
ADNFLE:	Autosomal Dominant Nocturnal Frontal Lobe Epilepsy
ADPEAF:	Autosomal Dominant Partial Epilepsy with Auditory features
<i>AKT3</i> :	V-akt murine thymoma viral oncogene homolog 3
BECOP:	Benign epilepsy of childhood with occipital paroxysms
BFNC:	Benign Familial Neonatal Convulsions
BFIS:	Benign Familial Infantile Seizures
BMEI:	Benign myoclonic epilepsy in infancy
BPEI:	Benign partial epilepsy in infancy
BRE:	Benign rolandic epilepsy
CAE:	Childhood absence epilepsy
CGH:	Comparative Genome Hybridization
<i>CHD2</i> :	Chromodomain helicase DNA binding protein 2
<i>CHRNA2</i> :	Neuronal acetylcholine receptor subunit alpha-2
<i>CHRNA4</i> :	Neuronal acetylcholine receptor subunit alpha-4
<i>CHRNA2</i> :	Neuronal acetylcholine receptor subunit beta-2
<i>CLCN2</i> :	Chloride channel, voltage-sensitive 2
CNV:	Copy Number Variant
CPS-Other:	Complex partial seizures originating from the parietal, frontal and occipital lobes
CPS-TLE:	Complex partial seizures, temporal lobe epilepsy
DNA:	Deoxyribonucleic acid
EEG:	Electro-encephalogram

<i>EFHC1</i> :	EF-hand domain (C-terminal) containing 1
FS:	Febrile seizures
FS+:	Febrile seizures plus
GABA:	Gamma-aminobutyric acid
<i>GABRA1</i> :	Gamma-aminobutyric acid A receptor alpha 1
<i>GABRD</i> :	Gamma-aminobutyric acid (GABA) A receptor, delta
<i>GABRG2</i> :	Gamma-aminobutyric acid receptor subunit gamma-2
GEFS+:	Generalized epilepsy with febrile seizures plus
GTCS:	Generalized tonic-clonic seizures
TCS:	Tonic-clonic seizures
TLE:	Temporal Lobe Epilepsy
IE:	Idiopathic epilepsy
ILAE:	International League Against Epilepsy
IS:	Infantile spasms
JAE:	Juvenile absence epilepsy
JME:	Juvenile myoclonic epilepsy
<i>KCND2</i> :	Potassium voltage-gated channel, Shal-related subfamily, member 2
<i>KCNQ2</i> :	Potassium voltage-gated channel, KQT-like subfamily member 2
<i>KCNQ3</i> :	Potassium voltage-gated channel, KQT-like subfamily member 3
<i>KCNMA1</i> :	Potassium large conductance calcium-activated channel, subfamily M, alpha member 1
<i>KCNT1</i> :	Potassium channel, subfamily T, member 1
LD:	Linkage disequilibrium

<i>LGII</i> :	Leucine-rich Glioma Inactivated 1 gene
LQTS:	Long QT syndrome
MAE:	Myoclonic-astatic epilepsy
MAF:	Minor allele frequency
<i>mTOR</i> :	Mechanistic target of rapamycin
NFLE:	Nocturnal frontal lobe epilepsy
NGS:	Next Generation Sequencing
NL:	Newfoundland
PCR:	Polymerase chain reaction
<i>PIK3CA</i> :	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PGE:	Primary generalized epilepsy
<i>SCN1A</i> :	Sodium channel subunit alpha-1
<i>SCN1B</i> :	Sodium channel subunit beta-1
<i>SCN2A</i> :	Sodium channel subunit alpha-2
<i>SCN2B</i> :	Sodium channel subunit beta-2
SISA:	Simplified method for Segregation Analysis
SMEI:	Severe Myoclonic Epilepsy of Infancy
SNP:	Single nucleotide polymorphism
<i>SYNGAP1</i> :	Synaptic Ras GTPase activating protein 1
Sz:	Seizures
<i>TBC1D24</i> :	TBC1 domain family, member 24
TDS:	Targeted deep sequencing
WES:	Whole exome sequencing
WGS:	Whole genome sequencing

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Co-authorship Statement

I hereby declare that this thesis incorporates material that is a result of joint research, as follows: The probands were identified by pediatric neurologists Drs. David Buckley and Muhammad Alam. Mrs. Sharon Penney, RN, requested telephone contact from parents of probands during clinic visits. Dr. Susan Moore designed the study protocol, HIC application, and accompanied me on home visits. Dr. Patrick Parfrey provided guidance in study design and execution. Dr. Terry Lynn Young provided training and expertise in molecular genetic laboratory techniques.

In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-authors was primarily through the provision of general guidance and expertise in identifying eligible subjects and diagnosing seizure disorders. I independently completed all recruitment of patients, interviews, chart reviews, data collection, pedigree construction, candidate gene selection, DNA sequencing, genotyping, data analysis, and facilitated the knowledge transfer to attending neurologists and patients.

I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

Declaration of Previous Publication

This thesis includes content from two original papers, on which I am the first author, that have been previously published in peer reviewed journals, as follows:

Title: Variable neurologic phenotype in a GEFS+ family with a novel mutation in SCN1A.
Authors: Mahoney, K., Moore, S. J., Buckley, D., Alam, M., Parfrey, P., Penney, S., Merner, N., Hodgkinson, K. & Young, T. L.
Publication details: Seizure, 2009, issue 18, pages 492-7.

Title: High incidence of pediatric idiopathic epilepsy is associated with familial and autosomal dominant disease in Eastern Newfoundland.
Authors: Mahoney K, Buckley D, Alam M, Penney S, Young TL, Parfrey P, Moore SJ.
Publication details: Epilepsy Research, 2012, issue 98(2-3), pages 140-7.

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University's School of Graduate Studies, some of the work presented in this thesis was achieved with the help of Drs. Susan Moore, David Buckley, Muhammad Alam, Patrick Parfrey, Terry-Lynn Young and Mrs. Sharon Penney.

Additional manuscripts published during part-time student studies:

- Title: Inherited deleterious variants in GALNT12 are associated with CRC susceptibility.
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- Title: A Newfoundland cohort of familial and sporadic idiopathic pulmonary fibrosis patients: clinical and genetic features.
Authors: Fernandez BA, Fox G, Bhatia R, Sala E, Noble B, Denic N, Fernandez D, Duguid N, Dohey A, Kamel F, Edwards L, Mahoney K, Stuckless S, Parfrey PS, Woods MO.
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- Title: Adult siblings with homozygous G6PC3 mutations expand our understanding of the severe congenital neutropenia type 4 (SCN4) phenotype.
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Chapter 1

Introduction and Overview

1 Introduction

1.1 Rationale

Epilepsy is a common, chronic neurological disorder marked by disturbed electrical rhythms of the central nervous system and typically manifested by recurrent convulsive attacks known as seizures. It creates a significant burden for the affected individual, their family and society. The lifetime prevalence of all epilepsies is 2-5% (Shorvon 1990; Hauser, Annegers et al. 1996) and the average incidence is approximately 40 to 80 per 100 000 children (Blom, Heijbel et al. 1978; Camfield, Camfield et al. 1996; Olafsson, Hauser et al. 1996; Zarrelli, Beghi et al. 1999; Freitag, May et al. 2001; Forsgren, Beghi et al. 2005; Theodore, Spencer et al. 2006). The heritability of epilepsy, as measured by the concordance rate between twin pairs, has been estimated to be 70-88%; that is, 70-88% of the liability to develop epilepsy can be accounted for by genetic factors (Kjeldsen, Kyvik et al. 2001). Family studies have shown a risk to the sibling of an affected person with epilepsy is approximately 2.5 to 4 fold greater than the general population risk (Annegers, Rocca et al. 1996).

Worldwide, the epilepsies are the second most common neurologic disorder, second only to headaches. In developing countries, the epilepsies are the most common neurologic disorder. In the United States, the epilepsies are second only to cerebrovascular disease and stroke. Of the 40 to 100 million persons with epilepsy worldwide, approximately 50% have generalized seizures. Among the generalized epilepsies, the most common are juvenile myoclonus epilepsy (JME) with 10% to 30% of cases, childhood absence epilepsy (CAE) with 5% to 15% of cases, and tonic-clonic seizures on awakening with 22% to 37% of cases (Delgado-Escueta, Medina et al. 1999). Moreover, certain types of seizures are unique to children, including absence, JME seizures, and most febrile seizures. Age-specific incidence studies of epilepsy have

demonstrated that the highest peak occurs in childhood (Kotsopoulos, van Merode et al. 2002). Thus, a disproportionately large number of children are burdened by epilepsy disorders.

The prognosis of patients with childhood epilepsy varies widely, depending on the type, frequency and severity of the individual's seizures or syndrome, as well as the patient's response to treatment, environmental factors and the severity of any underlying etiology. Many children with epilepsy respond well to treatment, achieve seizure control and experience no long term psychomotor deficits. However, there are also many cases of childhood epilepsy with intractable seizures despite various treatment regimens, as well as devastating long term cognitive and motor impairments, and even, in rare cases, death.

Although epilepsy research over the last two decades has uncovered many important findings into the molecular genetic basis of the common epilepsies, the prevalence and incidence have not decreased, and there is no known cure for the epilepsies. Researchers in the field of epilepsy are now cognizant that identifying mutations for the idiopathic generalized epilepsies could lead to curative treatment and eventual elimination of 25% to 35% of all epilepsies (Baulac and Baulac 2009; Michelucci, Pasini et al. 2012; Pandolfo 2012). Much research attention has begun to focus on mapping, positional cloning, and mutation analyses of the common generalized and partial epilepsies.

1.2 Objectives

- 1) To describe the incidence of pediatric IE on the Avalon Peninsula of Newfoundland, over a five year period.
- 2) To classify cases of pediatric IE based on clinical manifestations.

- 3) To determine the geographical distribution of pediatric IE, in the province of Newfoundland and Labrador.
- 4) To identify families likely to have a monogenic form of IE and to determine the underlying genetic etiology of these families.

1.3 Classification of epilepsy

Epilepsies are a diverse group of disorders with both shared and distinct features. Classification allows a coherent, systematic approach and serves as a universal language between physicians and health care providers all over the world. Classifying and describing seizure disorders with shared clinical and EEG (electroencephalography) features, plays an important role in the field of epilepsy research. Increasingly, classification systems are being refined as new information becomes available and our understanding of the etiology and presentation of each syndrome widens. Advances in neuroimaging and neurogenetics have been particularly important and are likely to fundamentally change our concepts of syndrome classification.

1.3.1 Seizure and epilepsy classification systems

The International League Against Epilepsy (ILAE) is a worldwide association of physicians and other health professionals which disseminates research findings on epilepsy and related seizure disorders through periodic publications interpreting relevant data pertaining to concepts and organizational systems to guide classification of seizures and epilepsies. In 1981 and 1989, respectively, the ILAE published guidelines for seizure and epilepsy classification and terminology. They recommended a system which classifies epilepsy syndromes according to presumed localization (partial, generalized, undetermined) and etiology (idiopathic, cryptogenic,

symptomatic). This became the most widely accepted and utilized classification system used worldwide (ILAE 1981; ILAE 1989).

The classification system divides seizures into those of partial onset and those of generalized onset, depending on whether the initial clinical manifestations indicate that one cortical region or both hemispheres are involved at the onset of the seizure. The undetermined group includes patients in which localization was not possible (ILAE 1989; Commission 1993; Engel 2006). A seizure is considered to be generalized when the EEG finding is consistent with a generalized pattern and when assessment of clinical symptoms provides no indication of an anatomic localization and no clinical evidence of partial onset.

When possible, three main **generalized** seizure subtypes are recognized:

- Generalized convulsive seizures with predominantly tonic, clonic, or tonic-clonic features;
- Generalized nonconvulsive seizures represented by absence seizures;
- Myoclonic seizures (Commission 1993).

In patients who have experienced several types of generalized seizures, each seizure type must be categorized.

Partial seizures are divided into simple partial seizures, in which a fully conscious state is retained, or complex partial seizures, in which consciousness is impaired. A seizure should be classified as partial when there is evidence of a clinical partial onset, regardless of whether the seizure is secondarily generalized. The first clinical signs of a seizure have a highly localizing value and result from the anatomic or functional neuronal activation of part of one hemisphere. When alertness and ability to interact appropriately with the environment is maintained, the seizure is classified as a simple partial seizure. When impairment of consciousness, amnesia, or confusion during or after a seizure is reported, the seizure is classified as a complex partial

seizure. When the distinction between simple and complex partial seizure cannot be made, from information provided by history or medical records, the seizure is classified as partial epileptic seizure of unknown type. When a patient has several types of partial seizures, each should be separately categorized. When a patient becomes secondarily generalized, the seizure is classified as a partial seizure, secondarily generalized (simple or complex) (ILAE 1989; Commission 1993; Commission 1993). This system has the advantage that seizures can be classified relatively easily and choice of an antiepileptic drug (AED) is dictated by seizure type.

Within the generalized and localized groups, there are further subdivisions into idiopathic, cryptogenic or symptomatic. Idiopathic epilepsies are generally genetic, and while many such syndromes have been described, advances in molecular genetics will undoubtedly reveal many more syndromes in the near future. Cryptogenic epilepsies are those in which an underlying environmental or somatic cause is suspected, but the etiology remains unknown. Epilepsies for which there is an underlying structural cause or major metabolic derangement are considered symptomatic (Edwards 2001). This classification is complex and many syndromes are not adequately defined. At initial presentation, it may not be possible to identify the syndrome. Only a small proportion of patients easily fit into specific syndromes while a large proportion fall into non-specific categories.

Prior to the guidelines published by the ILAE, international classification of seizure disorders relied on the use of clinical and EEG criteria. However, EEG is often unavailable or impractical. Such is the case in many field surveys, population polls, and in rural or undeveloped regions, worldwide. Therefore, in 1981, the ILAE established these classification criteria based predominantly on clinical data. The criteria was later revised in subsequent papers

published in 1989, 1993, and 2004 (Okuma 2004). Each subsequent article was meant to supplement the previous classification system.

In clinical practice, these guidelines are often useful to conceptualize epilepsy syndromes according to their usual age at presentation, which greatly facilitates syndrome identification in new patients and recognizes the age-related expression of many childhood epilepsies. However, classification problems exist for many pediatric epilepsy syndromes, particularly the epileptic encephalopathies of early infancy, the benign epilepsies of infancy and childhood, the myoclonic epilepsies of infancy and early childhood, and the idiopathic generalized epilepsies of childhood and adolescence (Duchowny and Harvey 1996). The clinical manifestation of epileptic seizures may vary widely from patient to patient, depending on the region of the brain involved. It is likely that further input from the fields of molecular genetics and neuroimaging will enable the classification of epilepsies to become more etiologically oriented and disease specific. Presently, classification of epilepsy diagnosis according to seizure phenotype is complex due to the diversity of clinical manifestations and the heterogeneity of seizures and other clinical manifestations within and between individuals and families.

There have been several attempts to update the concepts as new research findings emerged, but no new proposal was implemented until 2010 when the ILAE made several changes to the system (ILAE 1989; Engel 2006). These changes reflect the modifications to modern neuroimaging and our improved understanding of the molecular biology of epilepsy (Berg, Berkovic et al. 2010). As science progresses, health care professionals and researchers must evolve as well, in order to facilitate these advances. However, the retrospective design of the current study limited the ability for adaptation of new concepts and terminology. It would create significant selection bias to alter the inclusion and diagnostic criteria toward the end of the

study and apply these concepts to patients that have been previously enrolled, diagnosed, and classified. As a result, the modifications made by the ILAE in 2010 were considered to be additive to our current knowledge of epilepsy concepts, but we did not use the modifications suggested to alter the sample set. Figure 1 provides an overview of the primary classifications, including the traditional system, along with the recent modifications.

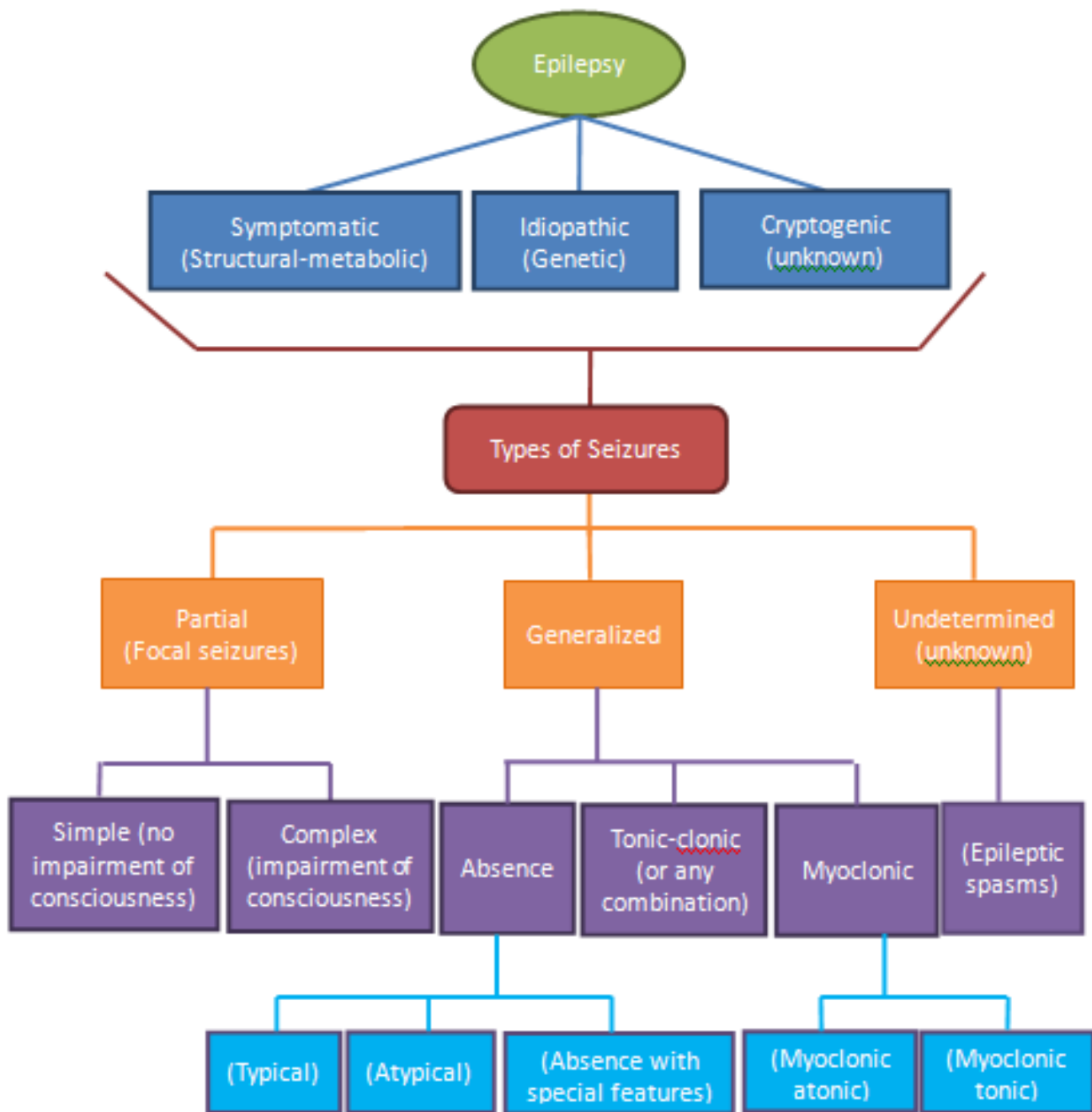


Figure 1. Overview of epilepsy classification systems. Modifications made by the ILAE in 2010 are shown in brackets.

1.3.2 Defining epilepsy and epileptic seizures

Defining the term epilepsy has been met with some disagreement due to the fact that epilepsy is not a singular disease entity but rather a variety of disorders reflecting underlying brain dysfunction resulting from multiple different causes. However, establishing definitions that can be broadly agreed upon is important for communication among medical professionals as well as determining parameters for those involved in legislation, disability benefits, driving regulations, workplace safety, education, etc. Consensus discussions amongst representatives of the ILAE have resulted in a definition of epilepsy that encompasses three elements; (1) history of at least one seizure, (2) enduring alteration in the brain that increases the likelihood of future seizures and (3) associated neurobiologic, cognitive, psychological, and social disturbances. These elements are articulated in the following definition; “**epilepsy** is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure.” (Fisher, van Emde Boas et al. 2005).

ILAE representatives also agreed upon a definition of epileptic seizure that encompasses three elements; (1) the mode of onset and termination, (2) clinical manifestations and (3) abnormal enhanced synchrony. The resulting definition is as follows; “an **epileptic seizure** is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain”. The clinical manifestation consists of sudden and transitory abnormal phenomena which may include alterations of consciousness, motor, sensory, autonomic, or psychic events, perceived by the patient or an observer (Commission 1993).

It is important to note that during an epileptic seizure, firing of neurons may involve inhibition as well as excitation. Thus, the key feature of neuronal networks generating an epileptic seizure involves abnormal enhanced synchrony of neurons, not simply excessive neuronal activity as once thought (Fisher, van Emde Boas et al. 2005).

However, as indicated by the definition of epilepsy, all seizures do not indicate epilepsy. Specifically, an individual may experience a seizure(s) but not fit the diagnostic criteria for epilepsy for multiple reasons. For example, an individual will not be diagnosed with epilepsy if they only experience one seizure, if they experience multiple seizures but they occur within the same 24-hour period, or if the seizures are provoked by some cause such as fever or some precipitating event, such as head trauma or a seizure-related disorder other than epilepsy.

The ILAE defines **idiopathic seizures** as unprovoked seizures occurring in the absence of a historical insult demonstrated to increase the risk of unprovoked seizures. The presence of an unexplained localized abnormality on neurologic examination (i.e. a reflex asymmetry) or identified in the diagnostic workup (i.e. focal slowing or focal spike on EEG examination, lesions of uncertain etiology identified on computed tomography or magnetic resonance imaging) are not excluded from this category. The ILAE reserves the term “idiopathic” for cases of presumed genetic origin. Thus, most cases in the idiopathic category would be further classified as cryptogenic upon assessment of neurological exams and/or sophisticated testing (Hauser, Annegers et al. 1991).

1.4 Pathophysiology of an epileptic seizure

An epileptic seizure is an episode of neurologic dysfunction in which abnormal neuronal firing is manifest clinically by changes in motor control, sensory perception, behavior, and/or

autonomic function. Epilepsy is the condition of recurrent spontaneous seizures arising from aberrant electrical activity within the brain. While anyone can experience a seizure under the appropriate pathophysiological conditions, epilepsy suggests an enduring alteration of brain function that facilitates seizure recurrence. Epileptogenesis is the process by which the normal brain becomes prone to epilepsy. The aberrant electrical activity that underlies epilepsy is the result of biochemical processes at the cellular level promoting neuronal hyperexcitability and neuronal hypersynchrony. However, a single neuron, discharging abnormally, is insufficient to produce a clinical seizure, which occurs only in the context of large neuronal networks. Cortical and several key subcortical structures are involved in generating a seizure. At a basic cellular level, an epileptic seizure may be understood to represent an imbalance between excitatory and inhibitory currents within neural circuits of the brain. A seizure occurs when a burst of electrical impulses (excitatory) in the brain exceed the threshold for normal functionality. The burst then spreads to neighbouring areas of the brain and creates an uncontrolled “storm” of electrical activity within the cortical nerve cells. These electrical impulses may be transmitted to the muscles causing twitching and/or convulsions.

1.5 Diagnosing epilepsy

A diagnosis of epilepsy is typically made after a patient is evaluated following one or more suspected seizures. Although the diagnostic techniques vary based upon the resources available, diagnosis for the current study was made following a thorough clinical work up involving family, medical, and seizure histories, and neurological examinations including electroencephalography (EEG) and magnetic resonance imaging (MRI).

1.5.1 Electroencephalography

Electroencephalography is a method of measuring electrical activity along the scalp. It uses an electroencephalograph to measure voltage fluctuations resulting from ionic current flows within the neurons of the brain and provide a record of the brain's ongoing electrical activity. Apart from the patient history and the neurological exam, the EEG is the most influential tool in the diagnosis of seizures and epilepsy. EEGs were first conducted in the latter parts of the 19th century. At this time they were utilized by just a few researchers, who performed their experiments on animal brains. It wasn't until the 1920s that the first EEG was conducted on a human subject. Since then, the EEG has been critical in both the research and clinical settings for assessments relating to the diagnosis of comas, brain tumours, stroke and encephalopathies; for the use in studying sleep and sleep disorders and particularly in neurology for diagnosing epilepsy (Swartz and Goldensohn 1998).

Simply stated, an EEG measures the electrical activity of the brain. However, this measurement is usually confined to the brain activity adjacent to the scalp, as EEG is not efficient at measuring the electric activity beneath the surface (cortex) of the brain. In other words, it has poor spatial resolution. Since neurons are electrically charged, particularly at the cortex, and many millions of neurons fire synchronously, an EEG can detect the electric potential of these cells. The EEG can identify the oscillations at different frequencies, that can be characteristic of particular types of brain activity (e.g. during walking or sleeping) or irregular activity (e.g. during seizures). These oscillations can have certain frequency ranges and spatial distributions. These differing outputs are highly informative as they not only indicate a seizure but may also provide insight regarding the type of seizure and particular form of epilepsy. The EEG tracing, with its record of electrical discharge, provides a record of activity in key areas of

the brain during the period of the test. Excessive discharge (of the type that may cause a seizure) may show up as a sharp spike or series of spikes; some patterns (such as the 3-per-second spike and wave of absence seizures) are unique to particular forms of epilepsy.

The encephalographer may try to evoke a seizure in order to obtain an EEG recording during the episode. Through photic stimulation, a flashing light is used to assess whether the patient is photosensitive, that is, if he or she will have a seizure in response to the stimulus of a flashing light. Hyperventilation (rapid over breathing) is another common trigger for seizures and is also a feature of an EEG assessment. Almost all patients with typical absence seizures who are not receiving antiepilepsy medication will have the characteristic 3-per-second spike wave EEG pattern during hyperventilation. Patients may be asked to go to sleep during the test because EEG abnormalities are more likely to show up during sleep. Various ictal (seizure) and interictal (between seizure) EEG patterns correspond to specific seizure types and types of epilepsy, although the correlation varies. While the EEG is almost always abnormal during a seizure, it may be normal between seizures. Thus, lack of interictal EEG abnormalities does not exclude a diagnosis of epilepsy. However, at some time, most epilepsy patients have abnormal EEG discharges. In contrast, some persons with EEGs that show epilepsy-like activity never have seizures. Thus physicians interpret EEG results within the context of other information they are gathering. See Figure 2 for a normal EEG pattern (A) and an EEG recording taken during an absence seizure showing the characteristic 3-per-second spike wave pattern (B).

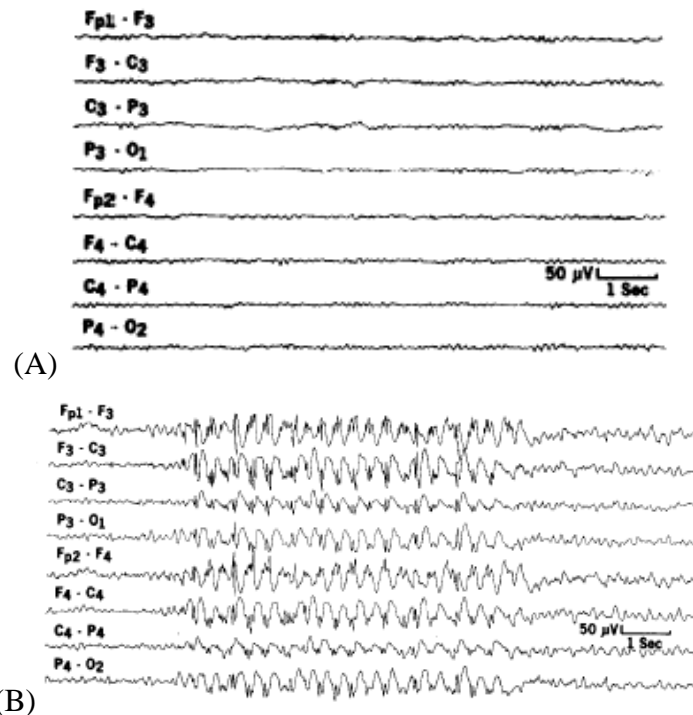


Figure 2. Normal and abnormal EEG recordings. (A) EEG recording of a normal brain showing no unusual activity (B) EEG recording of an absence seizure showing the distinctive 3-per-second spike and wave discharge. (www.epilepsy.com/EEG). Signal intensity measured in microvolts.

In neurology, EEG has also been particularly useful for distinguishing epileptic seizures from other types of events such as migraines, movement disorders and non-epileptic seizures. It can also be useful to characterize seizures for the purposes of treatment and to localize the part of the brain that is being affected by seizures, which is helpful for phenotyping or when brain surgery is warranted. The patient undergoing an EEG has electrodes placed on their scalp, which can be fastened in the form of individual electrodes or electrodes placed within a net or cap. The placement and names of electrodes is very specific and is specified by an international system in order to standardize between labs and clinics. The display of the EEG, mostly read by neurologists, represents the differences between two electrodes. As there are numerous electrodes (approximately 19 in low density arrays and several hundred in high-density arrays)

there are many possible representations of the EEG channels. The manner in which the encephalographer sets up the EEG is referred to as a montage. There are a number of standard montages, including sequential, referential and average reference (Lagerlund 2000).

Although the EEG is a very old technology and relatively primitive compared to other brain diagnostic technologies, such as fMRI (functional magnetic resonance imaging), PET (positron emission tomography), MEG (magnetoencephalography), or MRS (magnetic resonance spectroscopy), it remains very important in the clinical setting. It has the advantage of being relatively inexpensive to use and is highly mobile. Most importantly, it has a very high temporal resolution, meaning that it can measure on the order of milliseconds rather than seconds. As well, EEG measures brain electrical activity directly unlike the other techniques mentioned above which measure blood flow and metabolic activity which is an indirect marker of brain electrical activity. From a safety point of view, it is relatively non-invasive and does not involve exposure to high-density magnetic fields or radioligands. It has been, and continues to be, the “bread and butter” technology of clinical neurologists. Of course, there are numerous limitations of EEG too. As mentioned above, it has low spatial density and cannot identify specific parts of the brain where particular events of interest may be taking place and ultimately the signal-to-noise ratio is low, therefore sophisticated data analysis and interpretation is often needed (Swartz and Goldensohn 1998). However, for the current study design it was the most suitable method to determine brain activity patterns in our cohort of epileptic patients.

The Janeway neurology clinic utilizes a modern digital encephalogram which allows for use of any montage on any section of the EEG. The neurologists order EEGs for all suspected seizure patients. Photic stimulation and hyperventilation are routine and sleep deprived EEGs are done if the routine EEG is normal. Other forms of provocation are rare.

1.5.1.1 EEG characteristics

Frequency

Frequency refers to rhythmic repetitive activity (in Hz). The frequency of EEG activity can have different properties including: rhythmic (waves of approximately constant frequency), arrhythmic (no stable rhythms are present), and dysrhythmic (rhythms and/or patterns that characteristically appear in patient groups and rarely seen in healthy subjects). The primary human EEG wave patterns are delta, theta, alpha and beta (see Figure 3);

Delta has a frequency of 3 Hz or below. It tends to be the highest in amplitude and the slowest waves. It is normal as the dominant rhythm in infants up to one year and in stages 3 and 4 of sleep. It may occur focally with subcortical lesions and in general distribution with diffuse lesions, metabolic encephalopathy hydrocephalus or deep midline lesions. It is usually most prominent frontally in adults (e.g. FIRDA - Frontal Intermittent Rhythmic Delta) and posteriorly in children (e.g. OIRDA - Occipital Intermittent Rhythmic Delta).

Theta has a frequency of 3.5 to 7.5 Hz and is classified as "slow" activity. It is perfectly normal in children up to 13 years and in adults while sleeping but abnormal in awake adults. It can be seen as a manifestation of focal subcortical lesions. It can also be seen in generalized distribution in diffuse disorders such as metabolic encephalopathy or some instances of hydrocephalus.

Alpha has a frequency between 7.5 and 13 Hz. It is usually best seen in the posterior regions of the head on each side, being higher in amplitude on the dominant side. It appears when closing the eyes and relaxing, and disappears when opening the eyes or alerting by any mechanism (thinking, calculating). It is the major rhythm seen in normal relaxed adults. It is present during most of life especially after the age of thirteen.

Beta activity is "fast" activity. It has a frequency of 14 and greater Hz. It is usually seen on both sides in symmetrical distribution and is most evident frontally. It is accentuated by sedative-hypnotic drugs especially the benzodiazepines and the barbiturates. It may be absent or reduced in areas of cortical damage. It is generally regarded as a normal rhythm. It is the dominant rhythm in patients who are alert or anxious and have their eyes open.

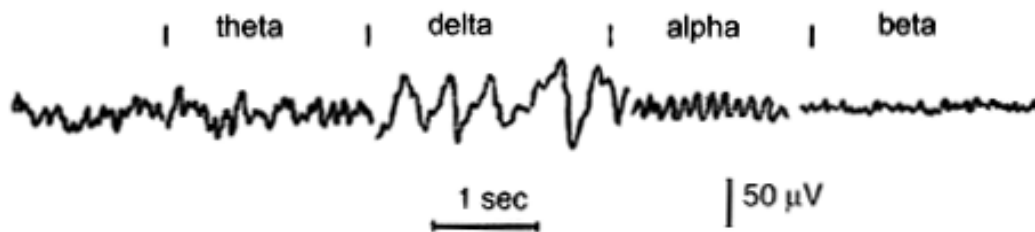


Figure 3. Representation of EEG recording showing the four main frequency patterns of human brain wave activity. (www.epilepsy.com)

Voltage

Voltage refers to the average voltage or peak voltage of EEG activity. Voltage values in an EEG are small and are measured in microvolts (μV). Descriptive terms associated with EEG voltage include: *attenuation* (reduction of amplitude resulting from decreased voltage), *hypersynchrony* (increase in voltage and regularity of rhythmic activity, or within the alpha, beta, or theta range), and *paroxysmal* (activity that emerges from the background with a rapid onset, reaching quite high voltage and ending with an abrupt return to lower voltage activity).

1.5.2 Magnetic resonance imaging

Magnetic resonance imaging (MRI) has been a mainstay of radiological diagnoses and disease staging for over 20 years. Based on using strong magnetic fields and radiowaves, an

MRI scanner forms a strong magnetic field around the area being imaged. By creating an oscillating magnetic field and then by using a beam of radio waves, the scanner excites hydrogen atoms in the cells of tissues that contain water. These hydrogen atoms in turn emit a radio signal that is detected by the scanner, which in turn is translated into an image by the computer. The contrast between different tissues is determined by the rate at which excited atoms return to the equilibrium state. By using a contrast agent that is administered into the blood stream, the image from the scanner can be enhanced. The importance of the MRI in medicine was highlighted when two scientists, Sir Peter Mansfield and Paul Lauterbur, were given the Nobel Prize in Physiology or Medicine in 2003, for their discoveries concerning MRI. Often MRI is used as an alternative for CT because it is more sensitive in determining small irregularities (e.g. tumours) and also because it is a safer alternative.

While the classification of seizure type still relies primarily on clinical history, observation and possibly EEG, MRI has been particularly valuable in detecting the abnormalities that cause specific types of epilepsy. Due to the sensitivity of MRI, it can identify scar tissue, areas of brain dysplasia, small brain tumours, blood vessel abnormalities and changes in white matter. Such abnormalities are often the cause of epileptic seizures. As well, MRI can help diagnose an epileptic syndrome. For example, if an MRI shows a structural lesion that is the likely source of the seizures, then a classification as partial in nature can be made. This often helps in determining the best treatment modality. However, some epilepsy syndromes, such as benign rolandic epilepsy, cannot be diagnosed with MRI because it is not linked to damage to the structure of the brain. In addition, an MRI can be informative for evaluating the likelihood that surgery would be effective in controlling seizures. It can identify mesial temporal sclerosis or cavernous angioma, which carry a favorable surgical prognosis, and malformations of cortical

development, which have a less favorable prognosis for postoperative seizure control. Finally, MRI can help identify the volume of tissue that needs to be removed during surgery.

In the present study, MRI was used to identify or rule out any underlying cortical abnormalities causing epilepsy in our study patients. Since some generalized EEG patterns are presumed to have an underlying genetic etiology, not all epilepsy patients have MRIs. At the Janeway neurology clinic, MRIs are ordered by neurologists if a focal feature is present on the EEG, even if benign rolandic epilepsy is suspected. Those with an identifiable cause on MRI were excluded from the idiopathic epilepsy group. As stated in the study inclusion criteria, patients must present with no known underlying cause of seizures aside from the presumed genetic predisposition.

1.6 Childhood Idiopathic Epilepsy Syndrome Classification:

Upon diagnosis of childhood idiopathic epilepsy, specific features of the clinical examination should help categorize patients into one of the following epileptic syndromes;

a) Idiopathic Generalized Epilepsies:

Primary generalized epilepsy (PGE) is a form of epilepsy characterized by generalized seizures with no apparent cause. Generalized seizures, as opposed to partial seizures, are a type of seizure that impairs consciousness and distorts the electrical activity of the whole or a larger portion of the brain which can be seen on EEG. Primary generalized epilepsy is the originally diagnosed condition, as opposed to secondary epilepsy, which occurs as a symptom of a known, diagnosed condition. PGEs typically have distinct patterns on EEG which are unique and provide evidence for the neurologist to presume there is no underlying etiology (ie. idiopathic).

Childhood absence epilepsy (CAE) is an idiopathic generalized epilepsy which occurs in otherwise normal children. The age of onset is between four and 10 years with peak age between five and seven years. Children with CAE have absence seizures which, although brief (approximately 4-20 seconds), they occur frequently, sometimes hundreds per day. The absence seizures of CAE involve abrupt and severe impairment of consciousness. Mild automatisms (repetitive unconscious gestures such as lip smacking, chewing, or swallowing) are frequent, but major motor involvement early in the course excludes this diagnosis. EEG recordings demonstrate characteristic 3Hz spike-wave discharges. Prognosis is excellent in well-defined cases of CAE with most patients out-grow their epilepsy by adolescence (Crunelli and Leresche 2002).

Juvenile absence epilepsy (JAE) is a relatively uncommon childhood seizure disorder with absence seizure onset typically occurring between 10 and 17 years of age. All patients experience absence seizures and many also experience GTCS (Generalized tonic-clonic seizures) and myoclonic attacks (Trinka, Baumgartner et al. 2004). Patients have normal intelligence and neurologic function. EEG recordings show generalized spike and wave discharges with normal background activity. Brain CT and MRI scans appear normal (Trinka, Baumgartner et al. 2004).

Juvenile myoclonic epilepsy (JME), also known as Janz syndrome, is a fairly common form of idiopathic generalized epilepsy, representing 5-10% of all epilepsies. It is characterized by the presence of absence seizures, myoclonic seizures and generalized tonic-clonic seizures. The hallmark characteristic of JME is the presence of myoclonic jerks that occur upon awakening.

These myoclonic jerks are the most prominent seizure type. This disorder typically first manifests itself between the ages of 12 and 18 with myoclonus occurring early in the morning. Patients typically do not out-grow this condition as it persists throughout life. Most patients also have tonic-clonic seizures and many also have absence seizures (Syvertsen, Markhus et al. 2012).

Generalized epilepsy with febrile seizures plus (GEFS+) is a diagnostic label coined by Scheffer and Berkovic in 1997 to describe a dominantly inherited epilepsy disorder associated with febrile and afebrile seizures. The phenotype is variable and can range from febrile seizures to severe intractable epilepsy with developmental delay in affected individuals within the same family. The prognosis also varies widely from seizure cessation with no long term deficits to intractable seizures with developmental delay (Scheffer and Berkovic 1997; Fujiwara, Sugawara et al. 2003).

Severe myoclonic epilepsy of infancy (SMEI), also called **Dravet syndrome**, is considered to be a genetic epileptic encephalopathy. This severe form of epilepsy appears during the first year of life (usually at around six months of age) with the onset of frequent febrile seizures. Beyond one year of age, other types of seizures typically arise, including absence, partial, atonic and often myoclonic seizures (involuntary muscle spasms). Status epilepticus (one continuous unrelenting seizure lasting longer than 30 minutes or recurrent seizures without regaining consciousness between seizures for greater than 30 minutes) may also occur. Early development is normal, with slowing and regression after one to two years of age. Children with Dravet syndrome typically experience an unfavorable prognosis with poor development of language and

motor skills, hyperactivity, and difficulty relating to others (Scheffer 2003; Dravet 2011; Dravet 2011 B).

Myoclonic astatic epilepsy (MAE) is a generalized idiopathic epilepsy characterized by the development of myoclonic seizures and/or myoclonic astatic seizures. Onset occurs between the ages of two and five. EEG recordings show regular and irregular bilaterally synchronous 2- to 3-Hz spike-waves and polyspike patterns with a 4- to 7-Hz background. The majority (84%) of affected children show normal development prior to seizures; the remainder show moderate psychomotor retardation mainly affecting speech. Boys (74%) are more often affected than girls (Doose and Baier 1987).

Benign myoclonic epilepsy in infancy (BMEI) is a form of idiopathic generalized epilepsy characterized by myoclonic seizures in the first three years of life in otherwise normal infants, and the lack of other seizure types except for rare simple febrile seizures. Myoclonic jerks (singular or clusters) occur in all cases while febrile seizures occur in approximately 10% of cases. Consciousness remains intact during seizures but mild clouding may occur during cluster of jerks. Males (at 66%) are more frequently affected than females (Ito, Oguni et al. 2012).

b) Idiopathic Partial Epilepsies:

Complex partial seizures (CPS) are epileptic seizures associated with bilateral cerebral hemisphere involvement and causes impairment of awareness or responsiveness, i.e. loss of consciousness. CPSs may arise from any lobe of the brain (Murro 2006) but most commonly arise from the mesial temporal lobe, particularly the amygdala, hippocampus,

and neocortical regions (Wolf 2008). CPSs occur when excessive and synchronous electrical brain activity causes impaired awareness and responsiveness and the abnormal electrical activity may or may not spread to the rest of the brain and cause a secondary generalized tonic-clonic seizure.

Benign rolandic epilepsy (BRE) is named after the rolandic area of the brain, which controls facial movement. BRE is the most common form of partial epilepsy in children, affecting both boys and girls, but is slightly more prevalent in boys. It usually starts between the ages of three and 12 years, and often stops around puberty (14-18 years of age). First presentation is typically a partial seizure with tonic-clonic activity of the lower face associated with drooling and impaired speech. Seizures commonly occur at night and may become secondarily generalized. The long-term prognosis for children with BRE is excellent with essentially all children entering remission by mid-adolescence; although, in some cases, specific difficulties with reading and language may persist. EEG recordings are typically easily recognizable with characteristic high-voltage sharp waves in the centrottemporal regions, which are activated with drowsiness and sleep (Wirrell 1998).

Benign epilepsy of childhood with occipital paroxysms (BECOP) / Panayiotopoulos syndrome describes benign partial epilepsies with focal origin and includes a subset of two syndromes: benign childhood epilepsy with centrottemporal spikes (BCECTS) and childhood epilepsy with occipital paroxysms (CEOP). Age of onset usually occurs in middle childhood (five to 11 years of age) but seizures can begin at any age from 15 months to 17 years. EEG recordings reveal abnormalities in the occipital region of the brain. About one third of children

have a family history of epilepsy and many have febrile seizures prior to BECOP onset. A 15-year follow-up prospective study indicated an excellent prognosis for most patients. The majority of children with this condition have normal neurological and learning abilities and brain scans appear normal (Panayiotopoulos 1989).

Benign Familial Neonatal Convulsions (BFNC) occur in otherwise healthy infants and are characterized most often by generalized or partial tonic-clonic seizures, normally within the first seven days of life and spontaneously remitting within the first two-three months. Other findings are a seizure-free interval between birth and the onset of seizures; normal physical examination and laboratory tests prior to, between, and after seizures; and absence of specific EEG findings. Approximately 50% to 70% of infants have a normal interictal EEG; about 25% demonstrate a specific EEG pattern known as *theta pointu alternant*; and a small percentage have focal, often rolandic, discharges or spikes. The EEG is usually normal by age 24 months. Psychomotor development is usually normal. About 10%-15% of individuals with BFNC develop epileptic seizures later in life (Bjerre and Corelius 1968; Mulley, Scheffer et al. 2003).

In the mid-1990s, a Newfoundland family with autosomal dominant BFNC was studied and the genetic investigation of their DNA eventually led to one of the first novel epilepsy gene discoveries. A gene that encodes a potassium channel subunit, *KCNQ2*, was first identified in this family in 1998 (Singh, Charlier et al. 1998).

Benign partial epilepsy in infancy (BPEI) is defined as epilepsies with complex partial seizures (CPS) or secondary generalized seizures (SGS), or both, compatible with the following characteristics: normal development before and after onset, no underlying disorders, normal

interictal EEGs, and good response to treatment (Okumura, Hayakawa et al. 1996). Seizure onset occurs in the first two years of life with an average age at onset of 5.9 months. Seizures typically persist for approximately three months and cease thereafter (Okumura, Hayakawa et al. 1996).

West Syndrome, also known as **Infantile Spasms (IS)**, are one of the most common and important epileptic encephalopathies of childhood. Onset of this syndrome occurs in infancy and consists of a unique seizure type (infantile spasms), a specific and characteristic EEG pattern (hypsarrhythmia), and psychomotor arrest or delay. IS is considered to be a “catastrophic epilepsy” due not only to the difficulty in attaining seizure control and in achieving normalization of the EEG, but primarily because, if untreated, affected infants almost uniformly deteriorate and develop severe developmental delay and mental retardation. However, early identification and aggressive treatment can potentially translate into a good prognosis and allow for a good neurodevelopmental outcome (Waternberg 2012). In an Icelandic epidemiological study of 214 patients diagnosed with infantile spasms, 31% died, 45% were retarded (IQ of 68-84), and 24% had a reasonably favorable outcome with an IQ of 85 or greater (Riikonen 2001).

1.7 Differential diagnosis

The differential diagnosis of epileptic and non-epileptic seizures or non-seizure episodes presents a number of challenges as the process of identifying the cause of seizures is quite diverse and complex. The occurrence of epileptiform EEG recordings in children without epilepsy has been well documented for decades. These patterns have been observed in patients with Cerebral Palsy (Gibbs, Gibbs et al. 1963), mental retardation (Trojaborg 1966), psychiatric

diseases (Zivin and Marsan 1968), behavioral disturbances (Lairy 1965), vegetative disturbances (Fois, Borgheresi et al. 1967), visual deficits (Jeavons 1964), and perceptive or praxic deficits (Berges, Harrison et al. 1968). There have also been documented correlations between EEG abnormalities and clinical disturbances. However, conclusions regarding the occurrence of such findings have remained difficult to draw as similar EEG findings have also been detected in subjects in which no clinical abnormalities can be found. In other words, these patients are considered completely normal from a clinical perspective, despite abnormal EEG recordings. An Italian study published in 1980 recorded EEG readings for 3726 children (aged 6-13 years) who were neurologically normal with no history of epileptic seizures and detected epileptiform abnormalities in 131 cases (3.5%). Follow-up studies were conducted on these patients with abnormal EEGs for a period of nine years. Spontaneous disappearance of EEG abnormalities by adolescence occurred in all but seven patients who developed epileptic seizures (Cavazzuti, Cappella et al. 1980). Using modern epidemiological methods, this data could be interpreted as a relative risk increase of developing seizures by adolescence of 1.05 (or 5.5%), given an abnormal EEG recording in childhood. This increase is not significantly greater than the population lifetime risk of 3-5%. Thus, EEG as a pre-symptomatic tool for diagnosis would drastically over-estimate the rate of children predisposed to seizures as it is highly sensitive but not highly specific resulting in a high false positive rate.

Furthermore, different diagnostic techniques for epilepsy provide different clues to the most probable diagnosis and each technique is not readily available in all areas. For example, long-term EEG monitoring with video recording (video EEG) is the strongest differential diagnostic tool available but it is also complex, costly and not available in all areas. Several other diagnostic techniques are regularly utilized in conjunction with or alternatively to video

EEG including; routine EEG, medical history variables, seizure semiology, provocative testing, and psychological and neuropsychological testing. Each technique has merit for diagnostic utility but are more successful as complementary diagnostic tools, rather than individual tests (Cragar, Berry et al. 2002).

In the current study, the differential diagnosis of an apparent seizure disorder was conducted by the neurologists in the Janeway Neurology Clinic. Several steps are involved in ruling out causes of alternate pathology with similar presentation, such as a cardiac arrhythmia, syncope, migraine, etc. The diagnoses were made utilizing thorough clinical work up, family history, observation, EEG, MRI, and electrocardiogram (ECG) when deemed appropriate in cases of diagnostic uncertainty.

1.7.1 Phenocopies

A phenocopy is an individual whose phenotype appears the same as another but the underlying etiology of the phenotype is different between them – usually one has a genetic cause of the phenotype, and the other, a non-genetic (environmental) cause. The possibility of phenocopies must be considered when diagnosing patients, particularly family members of affected patients that are affected by hearsay. When collecting data from study subjects, investigators are typically very thorough in determining that the correct diagnosis has been made. Medical and seizure history data are reviewed in addition to a complete clinical work up. Ideally, when conducting family studies, the same level of thoroughness should be employed when determining the correct diagnosis for family members. Unfortunately, this is often not the case due to restricted access to medical records, unavailability of technical diagnostic testing, or lack of resources. Thus, it is not unusual, depending on the phenotype and its frequency in the

general populace, for phenocopies to be present in family studies. The phenocopy rate is difficult to determine as it can often only be detected after the underlying genotype is established in the family.

Furthermore, each type of epilepsy may have phenocopies that are in fact, alternate types of epilepsy, benign seizures (such as febrile seizures), or seizure-associated disorders (such as neuronal ceroid lipofuscinosis). With much similarity between different seizure types, care must be taken to establish the correct diagnosis. Reducing the number of phenocopies present in family studies investigating the genetic etiology of disease is imperative to improving the quality of the outcomes of such studies.

1.8 *Epileptic encephalopathies*

An epileptic encephalopathy occurs when an epileptiform brain wave pattern impairs brain function. Certain types of seizures and epilepsy syndromes commonly cause an epileptic encephalopathy. These particular epilepsy syndromes are referred to as epileptic encephalopathies. In an epileptic encephalopathy, the epileptic activity itself contributes to cognitive and behavioral problems, beyond what is expected from the underlying cause. An epileptic encephalopathy can cause progressive academic, behavioral, and motor problems. Depending on the frequency and severity of the seizures, abundance of epileptiform activity and medications used to inhibit these seizures, patients with epileptic encephalopathies display a wide range of cognitive and behavioral problems. The most common epileptic encephalopathies typically begin in infancy and are defined by very frequent or severe seizures that contribute to a progressive disturbance of cerebral function. These syndromes include Dravet syndrome (also known as SMEI), Ohtahara syndrome (OS) and West syndrome (also known as Infantile

Spasms). Dravet and West syndrome are described above with the other childhood idiopathic epilepsy syndromes. However, OS has not been exclusively characterized as an idiopathic epilepsy due to the underlying cortical malformation identified in many cases. OS is a progressive epileptic encephalopathy which is characterized by early onset intractable tonic spasms within the first three months of life and affected infants have a burst suppression pattern on EEG (high voltage activity alternating with nearly flat suppression phases). These clinical findings in infancy evolve into West Syndrome between three and six months of age, with continuing epileptic encephalopathy between the ages of one and three years. It is an extremely debilitating progressive neurological disorder, involving intractable seizures and severe mental retardation. No single cause has been identified, although structural brain abnormalities are a major cause for this condition. Due to the underlying etiology attributed to a cortical malformation in many cases, OS has not been exclusively characterized as an idiopathic (genetic) epilepsy. Further research into the etiology of OS must be undertaken before it can be fully understood where this disorder should fall within the current classification system (Noh, Jane Tavyev Asher et al. 2012).

1.9 *Literature review*

Few population-based studies of the familial basis of childhood epilepsy have been undertaken. Two previous epidemiological studies of childhood epilepsy in northern Sweden and Hong Kong found that 9.6% and 9.7% of patients had a first degree relative with a history of epilepsy, respectively and an Estonian study found that 14.8% of patients had a family history of epilepsy amongst first and second degree relatives (Sidenvall, Forsgren et al. 1996; Beilmann, Napa et al. 1999; Beilmann, Napa et al. 1999; Kwong, Chak et al. 2001). In a population-based

Danish twin registry, the concordance rates of epilepsy in 11 900 twin pairs was found to be significantly higher in monozygotic twins than dizygotic twins (0.37 vs 0.08, $P < 0.01$) (Kjeldsen, Kyvik et al. 2001). Though several previous studies have included family history data (Sidenvall, Forsgren et al. 1993; Sidenvall, Forsgren et al. 1996; Beilmann, Napa et al. 1999; Beilmann, Napa et al. 1999; Freitag, May et al. 2001; Kwong, Chak et al. 2001; Mullins, O'Sullivan S et al. 2007), there are no previous reports giving detailed family histories in a population-based study.

1.9.1 Twin studies of epilepsy

Twin studies are one of the strongest study designs used in determining the genetic contribution to disease, including various forms of idiopathic epilepsy of childhood. In 2006, the results of an international, multicenter, collaborative twin study examining the etiology of benign rolandic epilepsy (BRE), were published in *Epilepsia*. BRE is a common childhood epilepsy syndrome that had previously been considered to be a genetic idiopathic partial epilepsy. The investigators reviewed twin registries from Australia, Denmark, the United States, and Norway (1952 twin pairs with a seizure history), to identify subjects with classic BRE and examine concordance rates. Of the eighteen twin pairs identified of whom at least one twin was diagnosed with BRE, all were discordant (pairwise concordance rate was 0.0). This data challenges the hypothesis that BRE is a genetic disorder and suggests that non-inherited factors are of major importance in the etiology of BRE and the mode of inheritance of BRE is much more complicated than initially believed (Vadlamudi, Kjeldsen et al. 2006). However, the limited number of twin pairs available reporting symptoms of BRE, lends questionability to the generalizability of the study results. A much larger sample of twins was enrolled in a

multicenter, population-based study which involved 47 626 twin pairs from the United States, Norway and Denmark. This study showed higher concordance rates of seizures between monozygotic pairs when compared to dizygotic pairs, across all subpopulation groups of different ages and populations (Kjeldsen, Corey et al. 2005). The latter study was more powerful due solely to the much larger sample size and the results are much more concordant with the vast body of literature supporting the important role of genetics in the development of epilepsy.

1.9.2 Population-based studies of epilepsy

Population-based studies allow researchers to obtain thorough epidemiologic data, implement and evaluate interventions, and provide care that is tailored to that population. Such studies improve the ability of the health care system to provide effective and efficient interventions that are consistent with the needs of the target population. Several population-based studies of epilepsy have been reported from various regions of the world over the last two decades (Callenbach, Geerts et al. 1998; Beilmann, Napa et al. 1999; Hesdorffer, Logroscino et al. 2011; Bhalla, Chea et al. 2012). The outcomes of interest were primarily epidemiological measures including incidence, prevalence, risk and classification of seizure types and epileptic syndromes. The current section presents the results of these studies chronologically and as relevant to the context of this thesis.

A longitudinal population-based study investigating the prevalence of epilepsy in Rochester, Minnesota determined that the age-adjusted prevalence per 1000 individuals, increased steadily from 2.7 in 1940 to 6.8 in 1980. Due to the vast improvements in neurological diagnostics during this time period, this marked increase could be somewhat attributable to the subsequent increase in diagnoses and ascertainment. This study also determined the prevalence

of epilepsy for a specific date in each of five decennial census years. At each of the five prevalence dates, for all prevalence cases, 60% had epilepsy manifest by partial seizures and 40% were generalized. Prevalence was higher for males than females for all. In terms of epilepsy duration and onset, of all prevalent cases in 1980, over 50% had epilepsy less than 10 years and had their first diagnosis in the first 20 years of life (Hauser, Annegers et al. 1991; Hauser, Annegers et al. 1993). In 2011, another publication from the same group in Rochester, Minnesota used retrospective data to assess the cumulative incidence and lifetime risk of developing epilepsy. The authors used incident cases of epilepsy occurring between 1960 and 1979 to calculate the lifetime risk to age 50 years is 1.6% and to age 80 is 3.0%. Cumulative incidence was 1.7% to age 50 and 3.4% to age 80 (Hesdorffer, Logroscino et al. 2011). This long-term study presented important information in determining epilepsy incidence and prevalence utilizing data from multiple decades. However, it provided no information on etiology and thus the number of idiopathic cases could not be derived for comparison to our study of the Newfoundland population.

A Dutch study of epilepsy in childhood followed 462 children with recurrent unprovoked seizures prospectively from 1988 to 1992 (Callenbach, Geerts et al. 1998). The purpose of the study was to examine the familial occurrence of epilepsy in children with newly diagnosed, multiple, unprovoked seizures. Seizures and epilepsy syndromes of probands were classified according to the ILAE classification system. Probands with at least one first-degree relative with epilepsy were selected. Seizures and syndromes of their relatives were classified via interviews. The results of seizure classifications demonstrated that 42% of probands had partial seizures, 57% had generalized seizures, and 1% had undetermined localization. The 47 (10.2%) children with at least one first-degree relative with epilepsy, less frequently had partial epilepsy (23%)

and more often had generalized epilepsy (77%), as compared with the total group of probands. Eighty-three percent (83%) of first-degree relatives with idiopathic or cryptogenic epilepsy had the same seizure type as the proband. This study concluded that in 10% of children with newly diagnosed epilepsy, the condition is familial. These children more frequently have generalized epilepsy syndromes as compared with children with a negative family history. These findings confirm the role of genetic factors in the pathogenesis of epilepsy (Callenbach, Geerts et al. 1998). This study provided comparable data from a population-based study. However, the criteria used in this study for inclusion in the “familial” group stated only that patients had at least one first degree relative with epilepsy. It provided no further information on pedigrees, possible inheritance patterns or the number of affected relatives. Thus, the proportion of families with inheritance patterns compatible with autosomal dominance was not provided.

In 1999, a team of researchers in Estonia reported a longitudinal, population-based study ascertaining all cases from every available source of medical care in seven counties of Estonia to determine the prevalence and phenotypic data of childhood epilepsy. Only cases of patients aged one month to 19 years with active epilepsy (defined as at least one seizure during the last five years, regardless of treatment) were included. The study concluded that the prevalence of childhood epilepsy was 5.8 per 1000, which was comparable to other Western countries. Generalized seizures were the predominating seizure type, and the primary cause was perinatal factors, including infections, drugs, and birth trauma (Beilmann, Napa et al. 1999). The strongest design element of this study was the longitudinal population-based approach which typically provides a broad picture of the epidemiological data for the given catchment area. However, since this study examined all cases of epilepsy, it provides no breakdown of epilepsy

by etiology. Therefore, it is difficult to draw comparisons to the incidence of genetic cases of childhood epilepsy in the study population of this thesis.

In a community-based study from Connecticut, of children with newly diagnosed epilepsy, diagnostic neuroimaging was examined for its efficacy in the evaluation of seizures and epilepsy. There is limited information about the current use of neuroimaging in the initial evaluation of pediatric epilepsy and about its yield during the initial diagnosis of epilepsy. The study investigators describe the patterns in the use and yield of diagnostic imaging in children with newly diagnosed epilepsy. Children were recruited when first diagnosed with epilepsy by participating physicians. Definitions for etiology and underlying epilepsy syndromes followed those published by the ILAE. Of 613 children, 488 (79.6%) had imaging: 388 (63.3%) had magnetic resonance imaging, 197 (32.1%) had computed tomography scans, and 97 (15.8%) had both. Half of children with idiopathic generalized epilepsy had imaging studies compared with 70% to 100% of children with other forms of epilepsy, depending on the specific type. Etiologically relevant abnormalities were found in 62 (12.7% of those imaged). Fourteen of these children had otherwise completely normal presentations and histories. Their abnormalities included tuberous sclerosis ($N = 4$), tumors ($N = 2$), an arteriovenous malformation later diagnosed as a tumor, a cavernous angioma, cerebral malformations ($N = 3$), and other abnormalities ($N = 5$). Thirteen of the 14 had partial seizures and 12 had focal EEG findings. Only one had neither. The study concluded that in children with newly diagnosed epilepsy, neuroimaging reveals a small but significant number of serious abnormalities not previously suspected. Most of these children have partial seizures or focal EEG abnormalities. Neuroimaging should be considered during the evaluation of children with newly diagnosed epilepsy, especially for those with neurologic deficits or partial seizures or focal EEG

abnormalities that are not part of an idiopathic localization-related epilepsy syndrome (Berg, Testa et al. 2000). Since neuroimaging is conducted for all children referred to the Janeway Neurology clinic in Newfoundland, this study was not referenced for testing indications, but rather for comparison of imaging results. The neuroimaging result data in the Connecticut study was thorough and provided specific information on the proportion of rare abnormalities which is useful for evaluating against the imaging data of the current study.

In a 2004 study conducted by the ILAE, 483 epileptic children attending a pediatric epilepsy clinic in a children's hospital in Bombay, India were classified according to the ILAE classification of epileptic seizures and epileptic syndromes. The predominant seizures were partial (53.6%), generalized (40.3%), and unclassifiable (6%). With respect to epileptic syndromes, 55.3% were partial, 27% were generalized, 13.5% were undetermined, and 4.1% were special syndromes. Although these results were similar in many respects to those of other reported studies, some differences were observed in the incidence of partial and generalized epileptic syndromes and their subgroups (idiopathic, symptomatic, and cryptogenic). The authors attributed these differences to differing age limits, methods of case ascertainment and inclusion criteria, genetic and environmental factors, variable interpretation of clinical and EEG features, and lack of facilities for investigation in developing countries. Despite various limitations, the investigators were able to classify most cases, and thus concluded that the ILAE classification can be used in developing countries so that comparisons can be made with other studies (Okuma 2004). This study used stringent criteria for diagnostic evaluation and was a useful proof of principle for the utility of the ILAE classification criteria that was employed in the study presented in this thesis.

The first population-based incidence study of epilepsy to describe syndrome classification from the outset was an Icelandic study published in 2005, which determined the annual incidence of a first unprovoked seizure to be 56.8 per 100 000 person-years and 33.3 per 100 000 person-years for epilepsy (recurrent unprovoked seizures). Idiopathic epilepsy syndromes were identified as the cause of 14% of cases (Olafsson, Ludvigsson et al. 2005). These findings were consistent with incidence studies from other developed countries. The population-based incidence data was directly comparable to that which we calculated for the current Newfoundland study. Also, the proportion of idiopathic epilepsy cases provides a frame of reference for the data presented herein, but without age-related data, it is difficult to draw comparisons to the childhood-based approach taken in the current study.

A 2012 population-based, case control study in Prey Veng, Cambodia identified 96 cases of epilepsy and matched 192 healthy controls. The purpose of this study was to identify attributable risk factors and determine measures of impact. Among this population, attributable risk to family history (defined as an affected family member of any degree) was 15%, other underlying disorders aside from seizures was 20% and eventful pregnancy / perinatal factors was 21%. This study also calculated the odds ratio of factors associated with epilepsy. Family history, in any degree relative, in cases versus controls gave an odds ratio of 2.9 ($p = 0.0018$) (Bhalla, Chea et al. 2012). As a measure of the association between the exposure and the outcome, odds ratio allows us to deduce that the odds of epilepsy cases having an affected family member is 2.9 times greater than the odds for controls. Although the outcome measures of this study differ from those which we were interested in for the Newfoundland study, it is of interest how they defined “family history”. Using the broad definition of a seizure history in any degree relative can lend to selection bias whereby cases with distant relatives could be classified as

having inherited forms of epilepsy when in fact the relative's seizure history is attributable to a different cause (phenocopy). The study made no attempt to adjust for this by further classifying cases with a family history into subcategories and therefore the results are difficult to generalize to other populations.

1.10 Genetic basis of epilepsy

Epilepsies are classified based on etiology as idiopathic, symptomatic or cryptogenic. Symptomatic and cryptogenic epilepsies result from known or suspected underlying disorders of the central nervous system, while in IE there is believed to be no underlying cause other than a hereditary predisposition. Approximately 40% to 60% of all cases are idiopathic and are presumed to have a predominantly genetic basis (Annegers, Rocca et al. 1996; Weissbecker, Elston et al. 1999; Andrade and Minassian 2007; Pandolfo 2012). The remainder are symptomatic, resulting from an underlying structural or metabolic cause, or cryptogenic, in which an underlying cause is suspected but not identified (ILAE 1989).

Family studies have shown that first degree relatives of an affected individual have a 2.5 to 4 fold greater risk of being affected than the general population, and twin studies have shown more than 40% monozygotic concordance rates (Annegers, Rocca et al. 1996; Weissbecker, Elston et al. 1999). Most cases of IE follow complex inheritance, involving the interaction of several genes and unknown environmental factors (Greenberg 1992; Berkovic and Mulley 1996; Berkovic, Howell et al. 1998; Berkovic, Mulley et al. 2006). Less frequently, IE can result from a single gene and follow a Mendelian pattern of inheritance. While a genetic contribution to the etiology of epilepsy is estimated to be present in about 40-60% of cases, Mendelian idiopathic epilepsies are individually rare, and are thought to account for no more than 1% of heritable

cases of epilepsy (Johnson, Robinson et al. 2011)(Gardiner 2000; Robinson and Gardiner 2004). These rare Mendelian cases are clinically and genetically heterogeneous with 18 causal genes (*KCNQ2*, *KCNQ3*, *KCND2*, *KCNT1*, *CHRNA4*, *CHRNA2*, *CHRNA2*, *CHRNA2*, *SCN1B*, *SCN1A*, *SCN2A*, *SCN2B*, *GABRG2*, *GABRD*, *GABRA1*, *CLCN2A*, *CACNB4*, *LGII* and *EFHC1*) identified to date (Berkovic, Mulley et al. 2006; Steinlein 2008; Baulac and Baulac 2009; Pandolfo 2012). All but two of the causative genes identified in IE encode components of neuronal ion channels (voltage-gated sodium, potassium, and chloride channels) or neurotransmitter receptors (acetylcholine nicotinic receptor and GABA_A receptor). A neuronal ion channel gene mutation was previously identified in the Province of Newfoundland and Labrador; a missense mutation in exon 6 of a potassium channel gene (*KCNQ2*) was previously shown to be associated with Benign Familial Neonatal Convulsions in a Newfoundland family (Singh, Charlier et al. 1998).

The first non-ion channel gene associated with Mendelian epilepsy, *LGII* (leucine-rich glioma inactivated 1), was identified as a cause of autosomal dominant partial epilepsy with auditory features (ADPEAF). A study at Columbia University (USA), conducted physical mapping of 10q24, genotyping, and candidate gene sequencing to identify *LGII* as the site of pathogenic mutations (four protein truncating variants and one missense variant) in five families with this rare form of epilepsy (Kalachikov, Evgrafov et al. 2002). The second such gene is *EFHC1* (EF-hand domain (C-terminal) containing 1) which has been associated with autosomal dominant juvenile myoclonic epilepsy (Suzuki, Delgado-Escueta et al. 2004). Table 1 provides a summary of all the genes that have been identified in IE, along with their chromosome position, associated phenotype, and typical mode of inheritance for each phenotype. The vast majority of the genes implicated to date have been identified in families with autosomal dominant IE.

1.10.1 Modes of inheritance

The genetic basis for epilepsy has primarily been studied in the context of clinically heterogeneous and genetically homogeneous families showing autosomal dominant inheritance. The characteristic clinical heterogeneity and genetic homogeneity is associated with the phenomena of one genetic variant causing disease and segregating in multiple family members, but the clinical manifestations can vary widely by individual. However, the vast majority of cases of IE likely follow some form of complex (polygenic) inheritance pattern. Although, in rare cases, the disorder can arise from a mutation in a single gene which segregates within families (monogenic inheritance) (Michelucci, Pasini et al. 2012). There is also limited evidence of other, non-Mendelian modes of inheritance including digenic and mitochondrial inheritance.

Digenic inheritance refers to mutations in two distinct genes interacting to cause a genetic phenotype or disease. This type of inheritance can be considered the simplest form of complex inheritance. A genetic linkage study of a French family with Temporal Lobe Epilepsy (TLE) reported evidence of digenic inheritance implicating chromosomes 18qter and 1q24-31. Haplotype analysis strengthened the hypothesis that two genes segregated with the disease in this family. However, no causative gene mutations were identified in these regions (Baulac, Picard et al. 2001). Linkage studies, such as this one, are an excellent tool to direct and supplement further sequencing studies; however, on their own, often lack sufficient strength to establish pathogenicity within chromosomal regions.

Mitochondrial inheritance refers to the process by which mitochondrial DNA (mtDNA) is inherited by offspring from the mother (maternally inherited). This single parent (uniparental) pattern of mtDNA inheritance occurs because mitochondria are inherited only in maternal ova and not in sperm. Therefore, the pattern of inheritance associated with alterations in mtDNA

will indicate both males and females being affected, but always being transmitted maternally. An affected male does not pass on his mitochondria to his children, so all his children will be unaffected.

Defective mitochondria have been implicated in numerous neurodegenerative diseases particularly Parkinson's disease and Alzheimer's disease. The role of mitochondria in the maintenance of neurons is well established especially in terms of autophagy (Ghavami, Yeganeh et al. 2012). Proper mitochondrial function is imperative for the survival of neurons as these cells are sensitive to the accumulation of damaged cellular components (e.g. membranes) that occur over time. Improperly functioning mitochondria are sometimes caused by mutated nuclear genes like *DJ-1*, *PINK1* and *PARKIN* – all of which have been implicated in Parkinson's disease. As well, cytochrome c, an important mitochondrial protein encoded by a nuclear gene, plays an important role in autophagy-mediated cell death. This protein, in conjunction with lysosomes, mediate efficient apoptosis without which aggregation and accumulation of cellular wastes occur which is especially toxic to neurons. That being said, there is not a great deal of evidence to date, linking neurodegeneration to the etiology of epilepsy. In some circumstances neuronal damage can occur due to epileptic seizures in the cases of epileptic encephalopathies. There have also been some examples of mutated mitochondrial genes causing epilepsy. Since the mitochondria are in high demand in the central nervous system and muscle, due to the high demand for energy in these tissues, it is reasonable to speculate that mutations in mitochondrial DNA could result in mitochondrial diseases of the brain. In fact, there are a group of mitochondrial encephalomyopathies (Goto, Nonaka et al. 1990). Epilepsy may be the presenting or a late onset feature of this group. Common mitochondrial epileptic syndromes include MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) and

MERRF (myoclonic epilepsy with ragged red fibers) syndromes. MELAS syndrome is genetically heterogeneous but is caused by mutations in *MT-TL1* (transfer RNA mitochondrial leucine 1 gene) in 80% of cases. The remaining known mutations are in other mitochondrial genes. MERRF, a syndrome characterized by myoclonus, epilepsy, ataxia, muscle weakness, hearing loss, and elevated serum lactate and pyruvate levels, is caused by a single point mutation in *MTTK* (transfer RNA mitochondrial lysine gene) in 80-90% of cases. Also, a very recent report (Almalki, Alston et al. 2013) has linked a missense mutation in the mitochondrial gene *FARS2*, which encodes phenylalanine-tRNA synthetase, with an epileptic phenotype. They showed that mutations in a single patient caused early-onset epilepsy and isolated complex-IV deficiency in muscle. Therefore, mitochondrial involvement has been shown to be important in neurodegenerative disease and in conditions where epilepsy is part of a larger spectrum of clinical manifestations. The importance of the mitochondrial genome in “non-syndromic” epilepsy has yet to be determined, however.

Futhermore, a recent Finnish study identified a novel mutation in the mitochondrial DNA cytochrome b gene (*MTCYB*) in epilepsy patients. The study’s inclusion criteria required patients to have two or more maternal relatives with a seizure history but made no distinctions based on etiology or type of epilepsy. Mitochondrial DNA sequencing identified a missense mutation, predicted to be deleterious, in five of 79 patients with epilepsy and four out of 403 population controls ($p = 0.0077$) (Soini, Moilanen et al. 2013).

1.10.2 Molecular Genetics of Epilepsy: Known epilepsy genes/mutations

The molecular genetic basis of epilepsy is known to be complex and the number of epilepsy-associated genes/mutations is continuing to grow partly due to recent advances in

genetic sequencing technologies. Many epilepsy syndromes are characterized by both locus and allelic heterogeneity. Locus heterogeneity refers to a single disorder caused by mutations in multiple genes at different chromosomal loci. For example, research to date has identified six genes which cause a GEFS+ phenotype. Similarly, allelic heterogeneity refers to a phenomenon in which different mutations at the same locus or gene cause a similar phenotype. Most epilepsy-associated genes have this characteristic whereby multiple mutations in the same gene have been identified and implicated to cause the same disorder. For example, dozens of mutations in *SCN1A* have been identified in SMEI patients. Additionally, pleiotropy, which refers to a gene (and mutations in that gene) influencing multiple, seemingly unrelated phenotypic traits, is characteristic of some epilepsy-associated genes. In fact, the first epilepsy-associated gene identified in humans, *CHRNA4*, has been shown to harbour a mutation which causes autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) in addition to a unique biopharmacological profile including reduced inhibition by carbamazepine and increased nicotine sensitivity (Hoda, Wanischek et al. 2009). Although, the molecular genetic basis of epilepsy is undoubtedly complex, many genetic contributors have been identified over the last two decades.

Currently, there are 18 known genes that contribute to the genetic component of monogenic epilepsy (Mulley, Scheffer et al. 2003; Meisler and Kearney 2005; Steinlein 2008; Pandolfo 2012). Amongst these 18 genes known to cause monogenic epilepsy, 16 are channelopathies and two are nonchannelopathies of unknown function. Seventeen of these 18 known monogenic disease genes have been determined by genome-wide linkage analysis from multiplex families with pedigrees suggestive of autosomal dominant inheritance. The final, and most recent gene to cause epilepsy when mutated, was identified by linkage analysis in addition

to whole-exome sequencing methods. The family ascertained for the latter study also presented with a highly penetrant form of autosomal dominant nocturnal frontal lobe epilepsy (Heron, Smith et al. 2013).

The first epilepsy gene, *CHRNA4*, was discovered in 1995 (Scheffer, Bhatia et al. 1995; Steinlein, Mulley et al. 1995). Mutations in this acetylcholine receptor subunit gene result in ADNFLE. This phenotype is characterized by partial seizures and this finding led the way to identifying several other monogenic genes for partial epilepsy discovered to date (Berkovic and Mulley 1996; Steinlein 2008; Pandolfo 2012).

Two potassium channel receptor subunit genes (*KCNQ2* and *KCNQ3*) have been found to harbour mutations in families presenting with Benign Familial Neonatal Convulsions (BFNC). *KCNQ2* was first identified in an autosomal dominant family residing in Terrenceville, Newfoundland in 1998 (Singh, Charlier et al. 1998). Since this discovery, other studies have published six different mutations in the *KCNQ2* gene, resulting in BFNC (Yalcin, Caglayan et al. 2007; Yum, Ko et al. 2010; Fister, Soltirovska-Salamon et al. 2012). Patients with BFNC typically have normal development and seizures that do not persist beyond the age of six months. More recently, *KCNQ2* has been implicated in a newly emerging phenotype with a much more damaging prognosis. A 2012 article in *Annals of Neurology* investigated whether *KCNQ2* mutations were present in 80 patients with unexplained neonatal seizures and associated psychomotor retardation. They identified seven different heterozygous *KCNQ2* mutations in 8 patients (10%); six of which arose *de novo*, one in which the patient's father was mosaic and had a less severe phenotype, and one in which the mother was not a carrier but the father's DNA was unavailable. In all of these eight patients, seizures began within the first week of life (two reportedly began in utero) and generally stopped by three years of age but the children had

profound to severe intellectual disability with motor impairment (Weckhuysen, Mandelstam et al.).

Recently another potassium channel gene, *KCNT1*, was identified to be the causative locus in an Australian family with a severe form of ADNFLE associated with major comorbidities including psychiatric and behavioral disorders, intellectual disabilities and a median number of 30 seizures per night (Derry, Heron et al. 2008; Heron, Smith et al. 2013). Further investigation of the *KCNT1* gene implicated two additional causative mutations segregating in two other families and a *de novo* mutation in a sporadic case with nocturnal frontal lobe epilepsy (NFLE) and psychiatric issues (Heron, Smith et al. 2013). The wide variability of phenotype in the epilepsy syndromes associated with potassium channel receptor subunit genes further substantiates the evidence that inherited forms of epilepsy are highly diverse and variable in their clinical presentation (clinically heterogeneous).

Four sodium channel receptor subunits (*SCN1A*, *SCN2A*, *SCN1B* and *SCN2B*) have been found to cause a recently described phenotype known as Generalized Epilepsy with Febrile Seizures Plus (GEFS+) (Wallace, Scheffer et al. 2002). The *SCN1B* gene is also implicated in Severe Myoclonic Epilepsy of Infancy (SMEI). GEFS+ is characterized by generalized afebrile seizures and febrile seizures that persist beyond the normal age range of six months to six years. SMEI is characterized by myoclonic seizures (jerking of the limbs, particularly the arms) in infancy and tends to present in patients with a family history of GEFS+. The sodium channel mutations resulting in these phenotypes differ in severity. A stop codon resulting in protein truncation and loss of function of that protein is associated with the SMEI phenotype, while mutations causing GEFS+ are typically missense mutations (Gardiner 2005).

Two gamma-aminobutyric acid (GABA) receptor subunit genes (*GABRA1* and *GABRG2*) have been found to be causative for CAE and JME. One chloride channel receptor subunit gene (*CLCN2*) has been found to cause several idiopathic generalized epilepsy subtypes including juvenile myoclonic epilepsy (Cossette, Liu et al. 2002). Mutations in a calcium channel beta-4 subunit gene (*CACNB4*) have also been found to cause juvenile myoclonic epilepsy in a single patient, generalized epilepsy in a German family, and episodic ataxia in a French Canadian family (Escayg, De Waard et al. 2000).

The two monogenic epilepsy genes that are non-channelopathies include an EF-hand motif gene (*EFHC1*) which causes JME and Leucine-rich Glioma Inactivated 1 gene (*LGII*) which causes Autosomal Dominant Partial Epilepsy with Auditory Features (ADPEAF). The exact function of these genes remains unknown (Turnbull, Kumar et al. 2008). However, it is believed that each of the five missense mutations of the *EFHC1* gene interfere with calcium channel function and mediate apoptotic activity (Medina, Suzuki et al. 2008).

Studies investigating alternate mechanisms of genetic contributors to the cause of epilepsy have also yielded some interesting findings. As described later, somatic mutations have been identified in genes previously associated with epilepsy. Epigenetic modifications have also been implicated in the etiology of epilepsy. Epigenetics has been defined as a set of self-perpetuating modifications on histone proteins or directly onto DNA that produce alterations in chromatin structure and alterations in patterns of gene expression (Lubin 2012). Recent evidence suggests that dysregulation of epigenetic mechanisms can cause disorders of the central nervous system. Histone modifications including acetylation and phosphorylation alterations have been implicated in epilepsy as well as Alzheimer's. However, the exact role these modifications play in epilepsy development remains unknown (Portela and Esteller 2010).

Gene by subtype	Chromosomal location	Phenotype associated with gene	Mode of inheritance	Reference
Voltage-gated sodium channel genes				
<i>SCN1A</i>	2q24	GEFS+ SMEI MAE	AD AD, <i>de novo</i> AD	(Escayg, MacDonald et al. 2000) (Claes, Del-Favero et al. 2001) (Ebach, Joos et al. 2005)
<i>SCN1B</i>	19q13.1	GEFS+ MAE	AD AD	(Wallace, Wang et al. 1998) (Sun, Zhang et al. 2008)
<i>SCN2B</i>	11q23	GEFS+	AD	(Wallace, Scheffer et al. 2002)
<i>SCN2A</i>	2q24	GEFS+ BFNIS SMEI	AD AD AD	(Sun, Zhang et al. 2008) (Heron, Crossland et al. 2002) (Kamiya, Kaneda et al. 2004)
Voltage-gated chloride channel genes				
<i>CLCN2A</i>	3q26	JME	AD	(Cossette, Liu et al. 2002)
Voltage-gated calcium channel genes				
<i>CACNB4</i>	2q22-q23	JME	AD	(Escayg, De Waard et al. 2000)
Voltage-gated potassium channel genes				
<i>KCNQ2</i>	20q13.3	BFNC	AD	(Singh, Charlier et al. 1998)
<i>KCNQ3</i>	8q24	BFNC	AD	(Singh, Westenskow et al. 2003)
<i>KCND2</i>	7q31	TLE	Single patient	(Singh, Ogiwara et al. 2006)
<i>KCNT1</i>	9q34.3	ADNFLE	AD	(Heron, Smith et al. 2012)
Neurotransmitter receptor genes				
<i>GABRG2</i>	5q31.1-q33.1	GEFS+	AD	(Baulac, Huberfeld et al. 2001)

		SMEI MAE CAE	AD AD AD	(Gennaro, Veggiotti et al. 2003) (Sun, Zhang et al. 2008) (Wallace, Marini et al. 2001)
<i>GABRD</i>	1p36.3	GEFS+	AD	(Sun, Zhang et al. 2008)
<i>GABRA1</i>	5q34-q35	JME CAE with JME	AD AD	(Cossette, Liu et al. 2002) (Wallace, Marini et al. 2001)
<i>CHRNA4</i>	20q13.3	ADNFLE	AD	(Steinlein, Mulley et al. 1995)
<i>CHRNA2</i>	8p21	ADNFLE	AD	(Aridon, Marini et al. 2006)
<i>CHRNA2</i>	1q21	ADNFLE	AD	(De Fusco, Becchetti et al. 2000)
Non-channelopathies				
<i>EFHC1</i>	6p12-p11	JME	AD	(Suzuki, Delgado-Escueta et al. 2004)
<i>LGII</i>	10q24	ADPEAF Familial lateral TLE	AD AD	(Kalachikov, Evgrafov et al. 2002) (Kalachikov, Evgrafov et al. 2002)
Alternate mechanisms				
<i>PIK3CA</i>	3q26	Hemimegalencephaly	<i>De novo</i>	(Poduri, Evrony et al. 2013)
<i>AKT3</i>	1q43	Hemimegalencephaly	<i>De novo</i>	(Poduri, Evrony et al. 2013)
<i>mTOR</i>	1p36	Hemimegalencephaly	<i>De novo</i>	(Poduri, Evrony et al. 2013)
<i>CHD2</i>	15q26.1	Epileptic encephalopathy	<i>De novo</i>	(Carvill, Heavin et al. 2013)
<i>SYNGAP1</i>	6p21.32	Epileptic encephalopathy	<i>De novo</i>	(Carvill, Heavin et al. 2013)
<i>TBC1D24</i>	16p13.3	Epileptic encephalopathy	Compound heterozygosity	(Milh, Falace et al. 2013)

Table 1. Genes identified in idiopathic epilepsy. AD, autosomal dominant.

1.10.3 Molecular Genetics of GEFS+

GEFS+ is genetically heterogeneous with five causative genes identified to date. The most widely reported is *SCN1A*, which encodes the $\alpha 1$ -subunit of a voltage-gated sodium channel and accounts for mutations in approximately 11% of previously described GEFS+ families (Escayg, MacDonald et al. 2000; Marini, Mei et al. 2007). Mutations in genes encoding the $\beta 1$ and αII subunits of voltage-gated sodium channels, *SCN1B* and *SCN2A*, can also cause GEFS+ (Wallace, Wang et al. 1998; Sugawara, Tsurubuchi et al. 2001). One splicing and six missense mutations associated with GEFS+ have been identified in *SCN1B* (Wallace, Wang et al. 1998; Audenaert, Claes et al. 2003; Scheffer, Harkin et al. 2007), and one missense mutation in the *SCN2A* gene has been associated with GEFS+ in a single patient (Sugawara, Tsurubuchi et al. 2001). In addition to mutations in subunits of the sodium channel, GEFS+ may also be associated with abnormalities in GABA neurotransmission, resulting from mutations in the *GABRG2* and *GABRD* genes, which encode subunits of the *GABA_A* receptor (Baulac, Huberfeld et al. 2001; Dibbens, Feng et al. 2004). Three missense mutations segregating in GEFS+ families have been reported in the *GABA_A* receptor, gamma 2 gene (Baulac, Huberfeld et al. 2001; Harkin, Bowser et al. 2002), while two have been reported in the *GABA_A* receptor, delta (*GABRD*) gene (Dibbens, Feng et al. 2004).

The most clinically relevant GEFS+ gene, *SCN1A*, has 26 exons and is highly conserved across species (Escayg, MacDonald et al. 2000). Mutations in *SCN1A* have been associated with a spectrum of epilepsy phenotypes, including severe myoclonic epilepsy of infancy (SMEI), GEFS+, and infantile spasms (IS) (Wallace, Hodgson et al. 2003; Harkin, McMahon et al. 2007). Of the 155 *SCN1A* mutations currently reported on the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk>), the vast majority are associated with SMEI, while 19 missense

mutations in the *SCN1A* gene have been associated with GEFS+ (Meisler and Kearney 2005; Mulley, Scheffer et al. 2005; Pineda-Trujillo, Carrizosa et al. 2005).

Recently, the phenotypic spectrum resulting from *SCN1A* mutations has been extended to include cryptogenic generalized and partial epilepsy, familial hemiplegic migraine, and familial sudden unexpected death in epilepsy (SUDEP) (Gargus and Tournay 2007; Harkin, McMahon et al. 2007; Hindocha, Nashef et al. 2008). Studies involving comprehensive clinical data on mutation carriers from multiplex families are necessary to determine the natural history and full clinical spectrum associated with the GEFS+ phenotype.

1.10.4 Channelopathies and epilepsy

There are over two dozen conditions caused by mutations in genes encoding channels, known as channelopathies, many of which have numerous channel genes causing the disorder, such as retinitis pigmentosa and Long QT syndrome (LQTS). Due to the almost ubiquitous distribution of ion channels in human tissues, mutations in the channelopathy genes can cause a wide array of clinical manifestations. Diseases such as asthma, autism, cystic fibrosis, nonsyndromic deafness, visual impairment, epilepsy, ataxia and cardiac sudden death have all been associated with deleterious variants in ion channel genes.

The vast majority of epilepsy-related genes are ion channel genes (see Table 1). Most notably, several sodium channel genes (*SCN1A*, *SCN1B*, and *SCN2A*) are known to cause epilepsy with a broad range of phenotypes. While mutations in the acetylcholine receptor genes (*CHRNA4*, *CHRNA2*, *CHRNA2*) cause a susceptibility to nocturnal frontal lobe epilepsy, characterized by nocturnal seizures occurring mostly during non-REM sleep. Mutations in calcium channel genes (*CACNA1A*, *CACNB4*, *CACNA1H*) have been associated with idiopathic

generalized epilepsy, juvenile myoclonic epilepsy, and childhood absence epilepsy. As well, mutations in potassium channel, chloride channel and GABA genes have all been associated with epileptic seizures.

Another well studied group of channelopathies are those related to sudden cardiac death (Webster and Berul 2013). Probably the best known and well-studied of the cardiac channelopathies is LQTS. There are at least 13 known ion channel genes that, when mutated, cause LQTS. Mutations in these genes can predispose the patient to arrhythmias. Other known cardiac channelopathies include short QT syndrome, Brugada syndrome, arrhythmogenic right ventricular cardiomyopathy, and catecholaminergic polymorphic ventricular tachycardia. In total there are approximately 40 known channel genes that cause cardiac arrest and/or sudden cardiac death.

Interestingly, in terms of this thesis, an increased mortality rate in epilepsy patients is partially caused by sudden unexpected death in epilepsy (SUDEP) which can be the result of cardiac arrhythmias in some epileptic patients (Velagapudi, Turagam et al. 2012). SUDEP accounts for 8%–17% of deaths in people with epilepsy and more often occurs in males. Although the risk of SUDEP is thought to be multifactorial, one of the contributors is having an underlying channel defect. In particular, mutations in genes that cause LQTS can also result in SUDEP. An analysis of 68 SUDEP cases (Tu, Bagnall et al. 2011) identified six novel variants in *KCNH2* and *SCN5A*. A pathogenic link to SUDEP was supported in two of these variants. However, subsequent studies have suggested that these variants are polymorphisms that may contribute to SUDEP. As well, there is limited data on the impact of *SCN1A* in the etiology of SUDEP. These three genes are well known to be responsible for LQTS. Of course, the risk of SUDEP in epilepsy patients, as noted above, is not exclusively determined by having a mutation

in a cardiac-related ion channel gene. The most prominent risk factors include the long-standing disease, poor seizure control, and antiepileptic therapy. Thus, SUDEP is an important consideration when clinically following high-risk epileptic patients, particularly with the ability to potentially prevent and treat cardiac arrhythmias.

As further evidence unfolds into the genetic underpinnings of SUDEP, it appears that the pathology is primarily multifactorial, and likely the result of multiple genes/mutations interacting to produce this devastating outcome.

1.10.5 Alternate genetic mechanisms of epilepsy

While most genetic conditions are considered to be directly inherited from one's parent and present in all somatic (body) cells, researchers are becoming increasingly aware of alternate mechanisms by which mutations can cause diseases, including epilepsy. For example, somatic mutations in three genes (*PIK3CA*, *AKT3*, and *mTOR*) have been shown to cause enlargement of one hemisphere of the brain (hemimegalencephaly) which is a malformation highly associated with epilepsy. Such somatic mutations are undetectable in the parents as they arise *de novo* (a latin expression meaning "from the beginning" which refers to a genetic mutation that neither parent possessed nor transmitted) (Poduri, Evrony et al. 2013). There have also been *de novo* mutations identified in *CHD2* and *SYNGAP1* that have been shown to cause epileptic encephalopathies. Multiple mutations in each of these genes accounted for the etiology in approximately 1% of the 500 cases of epileptic encephalopathy in this study. Interestingly, the vast majority of these mutations were highly deleterious (frameshift and nonsense mutations) and resulted in multiple seizure types and severe intellectual disability in all cases (Carvill, Heavin et al. 2013).

Rare cases of compound heterozygosity (e.g. recessive mode of inheritance) causing epileptic syndromes have also been reported. The term compound heterozygosity refers to the condition of two different mutations on separate alleles (one inherited from each parent) of the same gene causing a disorder. A 2013 study published in *Human Mutation* described two siblings with early onset epileptic encephalopathies attributed to compound heterozygous mutations in *TBC1D24*. The patients were born to healthy, unrelated parents and both children appeared to be healthy for the first month of life. Seizure onset and neurological regression began at four and five weeks of age and progressed rapidly with moderate to global brain atrophy by six months of age. Functional studies showed that the two *TBC1D24* mutations severely impair the expression of the TBC1D24 protein, which is critical to the maturation of neuronal circuits (Milh, Falace et al. 2013).

Somatic mosaicism has also been postulated to be a mechanism of epileptogenesis. Somatic mosaicism occurs when the somatic cells of the body are of more than one genotype. A commentary in *Brain* in 2008 presented the hypothesis that some portion of disorders of the central nervous system, such as epilepsy, could be caused by mosaicism generated in brain cells and tissues due to X-linked mutations. The theory was based upon research involving an X-linked neurological disorder (craniofacial dysplasia) with an inverse expression pattern; whereby females were affected and male carriers were unaffected (thus the opposite of the typical expression pattern seen in X-linked disorders) (Lindhout 2008). This expression pattern has also been observed in a rare epilepsy disorder known as epilepsy and mental retardation limited to females (EFMR) (Juberg and Hellman 1971). It is believed that disorders of the brain may be caused by somatic mosaicism because of the brain's immense interconnectivity which relies on the homogeneous make up of neurons and circuitry. The formation of genetically different tissue

within the central nervous system can create synaptic gaps in connectivity and alter the delicate excitation/inhibition balance of the brain. This theory has been demonstrated in mouse models but is yet to be proven in humans (Davy, Bush et al. 2006).

1.10.6 Chromosomal loci implicated in epilepsy

Six different chromosomal loci for common generalized epilepsies have been identified. These include two separate loci for JME on chromosomes 6p and 15q. Two separate loci are also present for pyknoleptic CAE, namely, CAE that evolves to JME on chromosome 1p and CAE with tonic-clonic seizures on chromosome 8q24 (Delgado-Escueta, Medina et al. 1999; Steinlein 2008). Two further putative gene locations have been implicated in idiopathic generalized epilepsy, namely tonic-clonic with generalized spike waves on chromosome 3p and generalized epilepsy with febrile seizures, JME, and absences on 8q24 (Pandolfo 2012). Table 2 provides a summary of all the loci that have been identified to date.

These chromosomal regions have not been substantiated by follow up studies identifying single epilepsy-associated genes with disease-causing mutations. Replication studies are required to lend further evidence and/or narrow these chromosomal regions and identify the positional candidate genes.

Chromosomal locus	Phenotype	Reference
6p11	JME	(Liu, Delgado-Escueta et al. 1996)
15q	JME	(Elmslie, Rees et al. 1997)
1p	CAE evolving to JME	(Delgado-Escueta, Medina et al. 1999)
8q24	CAE with tonic-clonic sz	(Fong, Shah et al. 1998)
3p	Tonic-clonic sz	(Delgado-Escueta, Medina et al. 1999)
8q24	Generalized epilepsies	(Delgado-Escueta, Medina et al. 1999)
19q12	BFIS	(Striano, Lispi et al. 2006)

Table 2. Chromosomal loci associated with epilepsy phenotypes. JME, Juvenile myoclonic epilepsy; CAE, childhood absence epilepsy; BFIS, Benign familial infantile seizures.

1.10.7 Approaches to disease gene discovery

In 2014, there are two major approaches to gene discovery for Mendelian diseases. Firstly, and the more traditional approach, is the candidate gene method to gene hunting which is ultimately carried out via direct Sanger DNA sequencing of previously disease-associated genes or by sequencing genes identified via linkage and/or haplotype analyses. Thus, a gene can be a candidate based on its chromosomal position (a positional candidate), the function of the protein for which it codes (a functional candidate), and/or its previous association with the disease of interest. Positional cloning and protein function studies was beyond the scope of the current study, thus candidates were primarily identified by their previous associations with epilepsy. For Mendelian conditions, a candidate gene is normally screened for mutations in one or more affected patients within a family. The presence or absence of a possibly pathogenic variant can later be tested in both affected and unaffected family members and/or healthy population controls. Healthy controls need to be analyzed to help prove that any variants postulated to be disease-causing are not rare non-pathogenic population variants.

The positional candidate approach often utilizes a statistical method called linkage analysis, and in concert with haplotype and segregation analyses. In order to perform these analyses patients and their families must be genotyped to determine if the gene of interest segregates on the same haplotype in all affected people within the family. This method utilizes molecular markers present in the genome, which may be Single Nucleotide Polymorphisms (SNPs) and/or microsatellites. Microsatellites are tandem repeating segments of one to six nucleotides, present throughout the genome and are used as molecular markers in genetic studies. SNPs and/or microsatellites that span the region of interest (which could be the entire genome) are selected and primers are designed to anneal to the surrounding sequence. Polymerase chain

reaction (PCR) experiments are then used to amplify these regions. PCR is a common laboratory technique used to amplify a single segment of DNA sequence to generate thousands to millions of copies, known as amplicons. The resulting amplicons are analyzed using a DNA sequencer and computer software to determine the number of repeats (for microsatellites) or the specific nucleotide (for SNPs) for each family member. This data is then assembled into haplotypes and analyzed on a pedigree to determine if the region of interest segregates with the disease in the families. Haplotype assembly takes advantage of the principals of genetic recombination. Chromosomes undergo crossovers during meiosis, which is the process of exchanging genetic information, usually between matching regions on homologous chromosomes. This exchange of genetic material is called genetic recombination. After a crossover event occurs, the newly formed chromosome is a recombinant. Alleles (alternate forms of the same locus) in close physical proximity to one another on the same chromosome, generally segregate together in blocks known as haplotypes (Keats, Sherman et al. 1991). Haplotype analysis is a very useful tool in defining a critical region within which a disease-causing gene is located. Within a haplotype, markers that do not have the same alleles among affected individuals due to recombination events can be used to further delineate the critical region containing the gene of interest. Thus, by taking advantage of this knowledge it is possible to track traits through families in a particular chromosome block, which can be identified by genetic markers that are physically adjacent to the disease gene. In order to determine if the genetic markers and the disease-causing mutation are linked, one must determine the phase (which parent passed on which allele to the child) and recombination points. Phase is difficult to determine when parents are homozygous for the particular allele. Such alleles are deemed uninformative for linkage as it is impossible to determine which parental copy was inherited. Alleles for which parents are

heterozygous must be utilized in order to determine phase and recombination status. These alleles are the basis of haplotype construction. Once a region of presumed linkage between a disease gene and genetic markers are identified, the subsequent step involves direct Sanger DNA sequencing of the coding segments of the genes within the segregating haplotype.

Over the past decade, the Sanger DNA sequencing method has been the most common approach to screening genes for causative variants. It may be used independently or in conjunction with linkage analysis. This method was introduced in 1977 by Fredrick Sanger and has since become the gold standard for generating or confirming sequencing data (Sanger, Nicklen et al. 1977). It involves the incorporation of deoxynucleotide triphosphates (dNTPs) with target DNA using primer sequences to determine nucleotide sequences. It is a tried and true technique that is considered the gold standard for mutation detection.

The second and much more recent strategy for disease gene hunting involves the latest technology in DNA sequencing methods, called Next Generation Sequencing (NGS). NGS refers to high-throughput DNA sequencing technologies whereby millions to billions of DNA strands can be sequenced in parallel, yielding substantially more throughput than Sanger sequencing. This technology has revolutionized how geneticists perform novel gene discovery programs and has begun to have clinical applications (Mardis 2008; Glusman 2013). NGS incorporates various platforms to produce high throughput data by way of simultaneous capture. The targeted region of interest depends on the platform selected (many platforms have now been developed by various companies) but they each utilize massively parallel sequencing strands. Multiple fragmented sequencing reads are assembled on the basis of their overlapping regions. Choice of platform depends on multiple criteria including price and data requirements. Whole genome sequencing (WGS) captures the entire genome, including untranslated and intergenic

regions. Whole exome sequencing (WES) captures only the exons and intronic boundaries of genes. Targeted deep sequencing (TDS) allows the user to select one or multiple chromosomal regions of interest and target the DNA sequence in only those particular areas. Thus, if a mutation in a specific gene has been previously associated or linked to epilepsy, it would be possible to sequence the entire coding and regulatory regions of that particular target gene in large samples (potentially thousands) of patients to determine if the same mutation or novel mutations in that gene are linked or associated with disease (Ferraro 2012). Each of these NGS methods has led to many breakthroughs in a plethora of genetic diseases (Glusman 2013). Epilepsy research has also recently benefited from NGS with the discovery of the *KCNT1* gene using WES technology (Heron, Smith et al. 2013). There are also potential clinical applications for these technologies in epilepsy research. With the large number of genes associated with inherited and sporadic forms of epilepsy, it may improve cost-effectiveness and efficiency to screen epilepsy patients for the known epilepsy genes using NGS technologies.

These technologies, however, carry their own unique set of limitations and challenges. The vast amount of data produced when employing NGS technologies has invoked an entire field of study developed to analyze and filter this data. These technologies can produce lists of hundreds of thousands of variants, of which, almost all are incidental findings. In the case of Mendelian disease, the researcher aims to identify one pathogenic variant amongst thousands. There are numerous software programs that incorporate bioinformatics data to identify the variants of highest biological impact. The data is reduced to incrementally shorter lists through various filtering techniques according to mutation severity, evolutionary conservation, functional pathways, and minor allele frequency in comparison populations. The short-list of potentially pathogenic variants must then be tested and confirmed using Sanger sequencing. Further

evidence of pathogenicity of a variant may then be gleaned through familial segregation studies, identification of the variant in unrelated patients, functional studies, etc. Although NGS technologies have allowed genetic studies access to the vast amount of information in the human genome, the analysis of this data is still in its early phases and further research in this area will allow for improved techniques for identifying pathogenic variants.

1.10.7.1 Determining the pathogenicity of sequence variants

Once sequencing data is generated, the task of determining the pathogenic nature of each variant must be diligently undertaken. In order to separate disease-causing mutations from common or rare variants that are not deleterious, various methods of filtering are utilized. There are currently several online DNA variation databases available to determine the frequency of variants (e.g. dbSNP, 1000 Genomes, UCSC Genome Browser, Ensembl, NHLBI). Some of the information in these databases consists of entire genome sequences of individuals, including the frequencies of alternate alleles at the same chromosomal positions. These are reported as minor allele frequencies (MAFs) and may include ancestry information for comparison purposes. For example, the current study was conducted on a Caucasian population and therefore used the MAFs for Caucasian populations as a comparison for previously reported variants. However, probably the most valuable sequence databases are actually in the laboratories of the centers that perform a high volume of NGS. These labs collate the data of all of the samples (anonymously) processed there, which is often many more genomes than are available publically. Furthermore, these databases, and locus-specific databases, can be used to determine if the variant is located in a coding or non-coding region of the gene, if the variant alters the amino acid for which it codes, and if the variant is located in a probable splice site position. There are also bioinformatic tools

available (e.g. SIFT, PolyPhen, Panther) which aid in determining if a variant may be deleterious. These publically available tools are just one of many instruments that should be utilized to determine the pathogenicity of variants. For many variants, pathogenicity is difficult to assess, but with the growing number of genomes being sequenced, there is hope that it will be less difficult in the future. Currently, using segregation data from affected and unaffected family members as well as population controls in addition to bioinformatic tools, MAFs and, ultimately, data from functional studies of the variants of interest, is all utilized to determine pathogenicity.

Further to generating segregation data for variants of interest, statistical methods can be employed to determine the probability that the segregation of a putative disease allele is statistically significant. A Simplified method for Segregation Analysis (SISA) is used to calculate the probability that a genetic variant cosegregates with the disease phenotype in a family by chance. It uses the level of disease expressivity in mutation carriers and non-carriers to determine the penetrance and expression of the variant in the family (Moller, Clark et al. 2010).

1.11 Benefits of studying the Newfoundland population

The population of Newfoundland and Labrador (NL) is predominantly of English and Irish extraction and has grown primarily by natural increase within small, isolated communities, resulting in multiple founder populations (Mannion, Memorial University of Newfoundland. Institute of Social and Economic Research. et al. 1978; Mannion and Memorial University of Newfoundland. Educational Television Centre. 1979; Bear, Nemec et al. 1987; Bear, Nemec et al. 1988; Rahman, Jones et al. 2003). Currently, the population of the province is approximately

509 200; about 50% of the population live in rural communities of fewer than 2500 inhabitants and approximately half the population (257 223) live in the Avalon Peninsula region (<http://www.statcan.gc.ca>). The Avalon Peninsula is a predominantly urban region and is the location of the province's capital city, St. John's (see Figure 4).

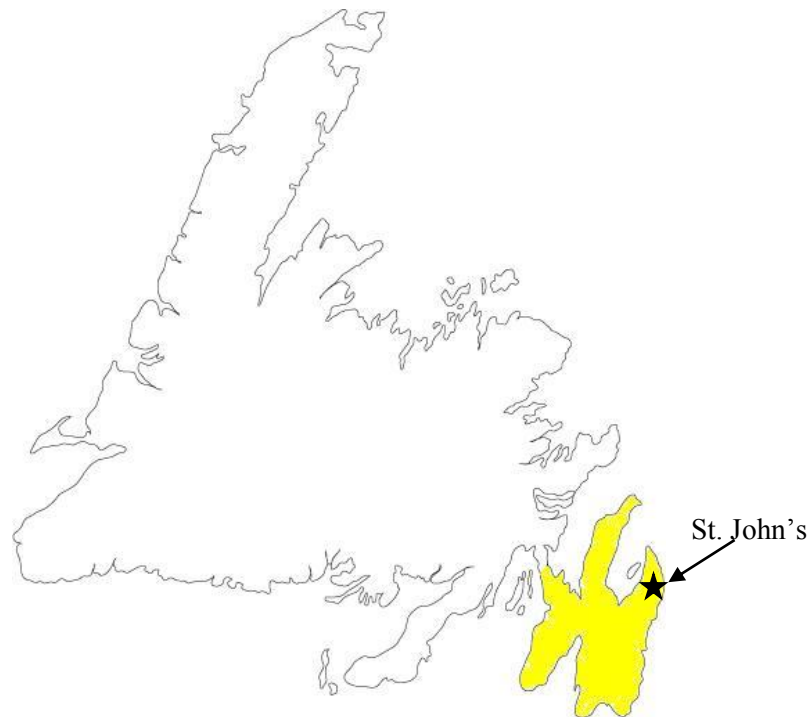


Figure 4. Map of Newfoundland indicating the most densely populated region and the capital city, St. John's. The Avalon Peninsula is highlighted in yellow.

The population of NL is genetically isolated, as is indicated by an overabundance of several monogenic disorders in which a founder effect (e.g. a genetic mutation which arose in a population many generations ago and now is responsible for disease in many individuals) has been observed. A provincial study of Bardet-Biedl Syndrome (BBS), a rare, multi-system autosomal recessive disorder, demonstrated that the condition occurred in multiple geographic isolates, but the genetic cause differed from region to region. In fact, six different genes were identified, in which 10 different mutations were identified as the cause of this syndrome (Webb,

Dicks et al. 2009). In autosomal dominant Lynch Syndrome, the genetic cause of this cancer syndrome also varied across geographic isolates on the island (Warden, Harnett et al. 2012).

The population of Newfoundland and Labrador presents a unique opportunity to study genetically inherited diseases such as idiopathic epilepsy. Like other genetic isolates that have arisen from a limited founder population, this province represents a valuable resource for identifying disease-related genes for monogenic disorders (Bear, Nemec et al. 1987; Bear, Nemec et al. 1988). In comparison to other developed regions, the relative genetic homogeneity of the Newfoundland population perpetuates genetic disease in such a manner that potentially allows molecular studies to yield more novel gene discoveries for numerous inherited diseases.

1.12 Study Protocol

The aim of the current study was to determine the clinical and genetic epidemiology of childhood IE in Newfoundland and Labrador. Specifically, this study was designed to determine the incidence of IE in the Avalon Peninsula region and the geographic distribution of sporadic and familial cases of childhood IE, as well as classify childhood IE in the province by clinical manifestations, identify families with IE compatible with likely monogenic inheritance, and to determine the underlying genetic etiology in these families.

1.12.1 Study Eligibility: Inclusion/exclusion criteria

Criteria for inclusion in the study included all children in Newfoundland and Labrador, aged 0 to 15 years during the seven-year study inception period from 1999-2006, with a history of two or more unprovoked seizures, more than 24 hours apart. Children with only febrile seizures, only one seizure, and/or known underlying seizure-associated disorders were excluded

from the study. Those diagnosed with a seizure-associated disorder (eg. neuronal ceroid lipofuscinosis, tuberous sclerosis, etc.) through the course of the study were excluded from further follow-up and analysis.

1.13 Thesis statement

This population-based study of the clinical and genetic epidemiologic characteristics of pediatric IE in Newfoundland creates a foundation of knowledge which is directly translated to improved patient care and also serves as a pilot for future studies to potentially identify novel epilepsy-associated gene(s) in this population.

Chapter 2

Methods

2 Methods

2.1 Study design and patient ascertainment

The current study is a provincial, population-based epidemiological study, designed to assess clinical and genetic features of childhood IE. The study was designed to research and measure several variables, both cross-sectionally and prospectively. Cross-sectional studies were employed to analyze the population of all children with IE for the entire province, while the prospective analyses utilized a representative subset of the population for one portion of the study and the entire population for the remainder of the study.

To determine the most accurate incidence of childhood IE, only the childhood population of the Avalon Peninsula was used. Within this subset of the NL population, it is estimated, by the physicians and staff at the Janeway Neurology Clinic, that the ascertainment of children with IE seen at this clinic approaches 100%. However, for the entire province, the ascertainment is approximately 95% (personal communication, Dr. David Buckley). Therefore, to obtain the most complete incidence data, only the Avalon Peninsula population was included in this portion of the study.

2.1.1 Cross-sectional analyses

Cross-sectional studies are a class of research methods that involve the observation of all of a population, or a representative subset, at one specific point in time. For this study, thorough medical records and a database of patient information maintained at the Janeway Pediatric Neurology Clinic allowed for epidemiologic analysis of the entire NL childhood population utilizing these baseline measures. Data was extracted from the database and medical records to compile patient information pertaining to gender, EEG recordings (classified as normal versus

abnormal), neuroimaging results (both MRI and CT scans), primary epilepsy diagnosis, classification by primary seizure type, active epilepsy status (occurrence of seizure(s) within the last one and five years), and anti-epileptic drug (AED) status.

2.1.2 Prospective study

Prospective studies follow a group or population of individuals over time to determine how certain factors affect outcomes of interest. The current study was designed to obtain patient data over several years to assess the etiology and level of impact of IE in this population of Newfoundland children. Patients were ascertained prospectively (all consecutive IE cases presenting to clinic) through the provincial pediatric neurology clinic at the Janeway Child Health Centre, the only children's hospital in the province. Inclusion criteria were as follows; residents of Newfoundland and Labrador, aged 0 to 15 years, with a clinical diagnosis of IE made between 1999 and 2006. IE was defined as two or more unprovoked, asymptomatic seizures occurring more than 24 hours apart in accordance with the International League Against Epilepsy (Commission 1993). Diagnoses were confirmed by the pediatric neurologists at the Janeway Child Health Centre (DB, MA). Children with a single unprovoked seizure, febrile seizures, or another precipitating or underlying cause discovered throughout the duration of the study were excluded from further investigation and removed from analyses. Children with psychomotor delay were not excluded from the study for having an underlying cause as the delays may be unrelated to the seizures or could be caused by the seizures themselves (as with epileptic encephalopathies).

Three-hundred ninety-five cases of childhood epilepsy, presenting to the neurology clinic between 1997 and 2006, were reviewed, yielding 121 cases (117 families; three with more than

one child presenting to clinic) who met eligibility criteria for the study. Ninety-seven of the 117 families were contacted and 86 agreed to participate, giving a response rate of 74%. Three attempts were made to reach the remaining 20 families by telephone and/or mail-out consent form. However, they chose not to respond and/or participate. Ascertainment bias was assessed by calculating the rate of familial disease in responders versus non-responders. The 11 families who did not participate in the family study either refused (n=6), relocated (n=3) or had social issues preventing participation (n=2). Figure 5 depicts the study design and ascertainment. The 86 participating families provided genealogical data for pedigree construction and answered medical and seizure history questionnaires (Appendix B). When necessary, and after informed consent was provided, medical charts were also reviewed for further medical history details.

Pedigree data was obtained by personal and/or phone interviews with the parent(s) or legal guardians to the patients. I gathered as much family history data as they could provide and, as needed, contacted other family members to obtain a more extensive history. I used this data to construct pedigrees, spanning at least three generations, and analyzed the disease segregation structure to determine the most probable mode of inheritance. Pedigree data was then transferred to digital format using Cyrillic pedigree drawing software (version 2.1). These pedigrees were then analyzed according to compatible inheritance patterns. Patients with no known family history were classified as sporadic cases. Patients with a family history in only one first degree relative or with affected relatives that were second degree or greater, were classified as having a family history of unknown type where the mode of inheritance was inconclusive. Patients with two or more affected first degree relatives, spanning at least two generations of the pedigrees, were classified as having a likely autosomal dominant inheritance pattern. Geographic data was used to determine distribution patterns and examine familial clustering. Blood samples were

obtained from 21 families (79 individuals) choosing to participate in the molecular phase of the study. Amongst the families with a positive family history, the mean number of affected relatives was calculated for two groups – those who participated in the molecular phase of the study and those who did not.

Patients and their family members were informed of any molecular findings that could improve the course of their medical treatment or have implications for genetic counseling. I provided the neurologist and research nurse with a mutation report for all individuals in which a pathogenic mutation was identified. The patients and family members were then notified of their result by the neurologist in the clinic and referred to genetic counseling for further follow up.

The study was approved by the Human Investigations Committee (HIC) of Memorial University (Study No. 4.15, see Appendices A and C for HIC application and study consent form).

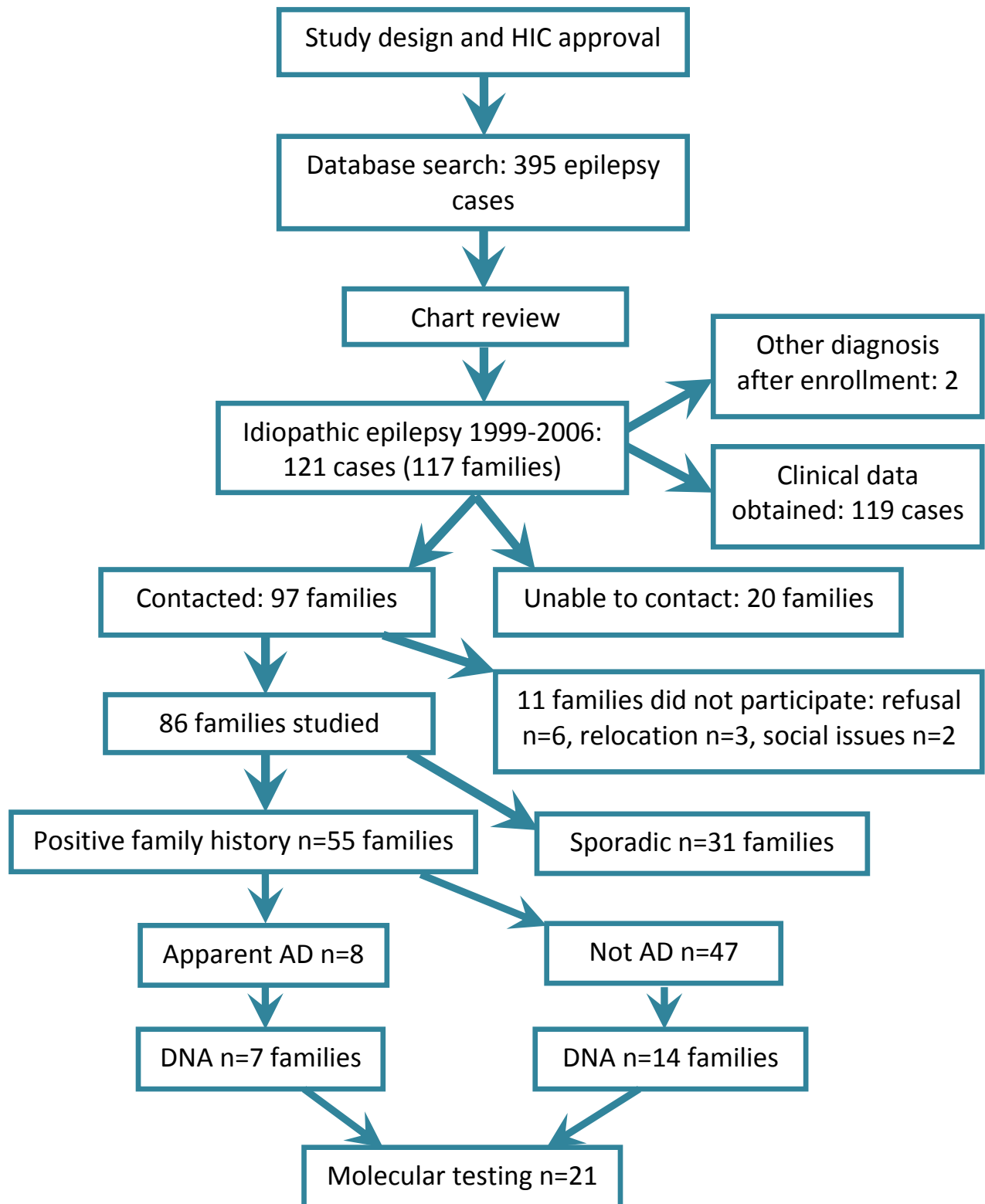


Figure 5. Flowchart depicting study design and ascertainment.

2.2 *Epidemiological measures*

A 5-year incidence of pediatric IE for the Avalon Peninsula was calculated retrospectively, using population data from Statistics Canada. This region was chosen because all childhood cases of IE presenting to family physicians, clinics and emergency rooms on the Avalon Peninsula are referred to the Janeway Neurology Clinic (personal communication, D Buckley). Children living in all other regions of the province are typically treated solely by their family physician or may be referred to the nearest health clinic. The number of new incident cases on the Avalon Peninsula, over a 5-year period was divided by the average population of children, aged 0-15 years, living in the region during that period. An incident case of IE was defined as a person diagnosed with epilepsy in the previous five years, regardless of antiepileptic drug treatment or symptom remission.

A database maintained by the neurologists and research nurses at the Janeway Pediatric Neurology Clinic was used to obtain case status, age at onset, age at diagnosis, seizure type(s), medication(s), family history status, and epilepsy syndrome diagnosis. Epilepsy diagnoses, as written in patient charts by the attending neurologist, are coded based on the primary epilepsy diagnosis and the data is then entered into the database by a research nurse. Definitions for etiological categories were based on the most recent criteria published by the ILAE Commission on Epidemiology and Prognosis (Commission 1993).

Data on family history, medical history and investigations including neuroimaging (MRI and CT brain scans) and EEG reports were obtained from medical chart reviews and interviews performed during clinic visits, over the phone, and during in-home visits. A positive family history of IE was defined as the occurrence of epilepsy in at least one first or second degree relative of the proband (Mullins, O'Sullivan S et al. 2007). Positive family history cases were

further classified as being compatible with autosomal dominant or other (non-autosomal dominant) familial inheritance. Compatibility with AD inheritance was defined as the presence of IE in at least two generations and in at least three relatives, one of whom is a first degree relative of the other two. The remaining cases in the positive family history group that did not fulfill the criteria for AD inheritance were classified as having non-AD familial disease.

2.2.1 *Informed consent*

For both the family/medical history and DNA extraction phases of this study, informed consent was obtained from parents or legal guardians. When patients were of literacy age, they were briefed on the ethical considerations of the study and were also asked to sign informed consent forms.

2.3 *Molecular method selection*

The molecular approach was a candidate gene screen based on phenotypic features in multiplex families with apparent AD inheritance. Phenotype data was carefully examined, for both the probands and family members of these families, and used to select the highest priority candidate genes, based on previously published phenotype-genotype associations. Linkage analysis was selected as the initial candidate gene approach to screen family A. Both affected and unaffected relatives were selected for genotyping to enable phasing of polymorphic markers. The subsequent gene screening method selected after linkage analysis was direct Sanger sequencing of the coding exons and splice sites of candidate genes.

2.3.1 *Molecular methods*

Those families choosing to provide DNA for molecular analysis were all entered into the molecular phase of this study (n=21). Eight candidate genes were selected based on previous associations with IE syndromes similar to those in the current study. The primary consideration involved in candidate gene selection and prioritization was the frequency of occurrence of associations between the gene and phenotype of interest in previously reported studies. *SCN1A* was prioritized as it is the most common childhood IE-associated gene. *KCNQ2* was also prioritized as it was the only gene to be associated with epilepsy in Newfoundland at the time the study was undertaken (Singh, Charlier et al. 1998). Genomic DNA samples from 21 probands (7 with a likely AD family history) were sequenced for all 26 exons of *SCN1A* (NCBI accession no. **NM_006920**) and all 16 exons of *KCNQ2* (NCBI accession no. **NM_172107**). The largest isoforms of each gene were selected. Blood samples were obtained from affected children and their relatives (affected and unaffected) and genomic DNA was extracted from peripheral blood using standard salting out methods (see Appendix G) (Miller, Dykes et al. 1988). Direct bidirectional DNA sequencing of the *SCN1A* gene and the *KCNQ2* gene was conducted for 74 samples from 21 families. Sequencing traces were analyzed with Mutation Surveyor v.3.20 software. Variants predicted to be deleterious based upon online bioinformatic software searches, were screened in 190 healthy population control alleles. DNA samples from probands of 20 families were then sequenced for the largest isoforms of the remaining six candidate genes: *SCN2A* (NCBI accession no. **NM_021007**), *SCN1B* (NCBI accession no. **NM_199037.2**), *KCNQ2* (NCBI accession no. **NM_004519.3**), *GABRA1* (NCBI accession no. **NM_000816.3**), *GABRG2* (NCBI accession no. **NM_000816.3**) and *KCNQ3* (NCBI accession no. **NM_004519.3**) (See Appendix F). Sequencing primers were custom designed for PCR amplification of all coding exons, untranslated regions, and intron-exon boundaries using

Primer3 software (<http://primer3.sourceforge.net/> and <http://frodo.wi.mit.edu/>). Standard PCR reactions were optimized and performed for each primer set. Primer sequences and optimized PCR conditions are provided in Appendices C & D. PCR products were confirmed on 1% agarose gel using ethidium bromide. Products were then purified using a Sephadryl spin column purification method. Purified samples were sequenced using BigDye Terminator v.3.1 Cycle Sequencing Kit and run on an ABI 3130xl Sequencer (Applied Biosystems, California, USA). Forward and reverse sequencing traces were aligned with reference sequences (from UCSC Genome Browser) and inspected manually for quality control and analyzed using Mutation Surveyor v.3.20, Sequencing Analysis 5.2, and Sequencher 4.10.1. All variants were recorded and searched online using variant databases (NCBI (<http://www.ncbi.nlm.nih.gov/>) and Ensembl (<http://www.ensembl.org/index.html>)) to determine allele frequencies and population demographics. The frequencies of rare variants (minor allele frequency of less than five percent) were tested against 190 ethnically matched healthy population control alleles. Several online bioinformatics tools (PolyPhen (genetics.bwh.harvard.edu/pph2/), SIFT (<http://sift.jcvi.org/>), and PANTHER (<http://www.pantherdb.org/>)) were used to predict the effects of variants on protein function and evolutionary conservation. Information on specific analysis performed is provided in Table 4 (page 92).

2.4 *Further investigation of most severely affected family*

2.4.1 *Clinical Features of Family A*

Family A (Figure 10) represented the most severely affected family with AD-compatible IE, enrolled in the study. The proband of family A was ascertained from a registry of all epilepsy patients treated at the Pediatric Neurology Clinic in the Janeway Child Health Centre

(St. John's, NL, Canada). The pedigree was extended through family visits and interviews and further clinical data was obtained from EEG recordings, standardized seizure and medical questionnaires (Appendix B), and medical records. Epilepsy diagnoses for family members were made by pediatric neurologists (DB, MA) using the ILAE classification system (Commission 1993). Informed consent was obtained from all participants or parents/legal guardians.

2.4.2 Genetic Analysis of Family A

The predominant phenotype expressed throughout Family A was determined to be consistent with the GEFS+ spectrum, via chart reviews and medical/seizure history questionnaires of patients and family members. PubMed was used to search previously published families with AD forms of GEFS+ with known mutations. The most common GEFS+-associated gene, *SCN1A*, was prioritized as the most likely candidate gene to harbor a disease-causing mutation. Genotyping was selected as the first method of genetic analysis to determine if *SCN1A* segregated with the disease throughout Family A. Sixteen family members (10 affected, six unaffected) were genotyped for four microsatellite markers (D2S2330, D2S156, D2S399 and D2S382) and two intragenic SNPs spanning the *SCN1A* gene on chromosome 2q24 (Figure 11). Genotyping primer sequences for the four microsatellite markers were extracted from UCSC Genome Browser website (<http://genome.ucsc.edu/>). The forward microsatellite primers were tagged with a fluorescent blue label known as 6-FAM. Sequencing primers were custom designed to amplify all 26 exons, intron/exon boundaries and 5' and 3' untranslated regions (UTRs) of *SCN1A*. Standard PCR reactions were performed with a reaction volume of 25ul containing 5ul of 3.75 M betaine, 2.5ul of 10x PCR buffer, 2.5ul of 2mM dNTPs, 0.75ul of 50mM MgCl₂, 0.2ul of 500U Taq Polymerase, 1ul each of 10uM forward and reverse primer,

and 1ul of genomic DNA at 100ng/ul. Touch-down PCR conditions were as follows: denature at 95°C for 5 min, followed by 5 cycles of 95°C for 30s, annealing at 64°C for 30s (decreasing by 0.5°C/ cycle), and extension at 72°C for 30s. This step was followed by 30 cycles of 95°C for 30s, 54°C for 30s, 72°C for 30s, a final extension at 72°C for 7 min, and held at 4°C.

Microsatellite samples were prepared for fragment analysis by adding 0.5ul of a labeled size standard (GeneScan 500 LIZ Size Standard) to 10ul of diluted (1/10), fluorescently labeled, PCR fragments. Samples were analyzed on an ABI 3130xl (Applied Biosystems) using the Fragment Analysis program. GeneMapper v4.0 software was used to create standard curves with peaks corresponding to the base-pair lengths of the microsatellite tandem repeats of each sample. Repeat sizes were then measured and recorded. Blinding of sample identifiers was implemented to eliminate interpreter bias.

Primers designed to surround regions with one or more SNPs spanning the *SCN1A* gene were used to amplify those DNA segments. The samples were then sequenced using BigDye Terminator v.3.1 Cycle Sequencing Kit and run on an ABI 3130xl (Applied Biosystems) using the sequencing analysis program. Sequence traces were inspected manually for quality control and analyzed using Mutation Surveyor v.3.20. The effects of variants were predicted using online bioinformatics tools (Ensembl, PolyPhen, SIFT, PANTHER and BDGP: Splice Site Prediction by Neural Network). Further data on specific protocols and analyses are provided in Appendices D and E.

2.4.3 Segregation and penetrance calculations in Family A

Segregation and penetrance of a putative pathogenic variant in Family A were determined using a **S**implified method for **S**egregation **A**nalyses (SISA). This method calculates the

probability that cosegregation of the disease and the genetic variant is due to chance using the following equation; $(1/2)^n$ where n is equal to the number of individuals in the pedigree which carry the mutation minus 1. Penetrance can then be calculated by dividing the number of affected variant carriers by the total number of variant carriers (Moller, Clark et al. 2010). These statistical measures of genotype-phenotype association can be used to determine the level of significance and confidence we have when postulating that a mutation is pathogenic in a given family. These data, in conjunction with the other types of evidence discussed here, offer researchers standardized numeric information on probability of variant and disease association.

Chapter 3

Results

3 Results

3.1 Epidemiology

3.1.1 Incidence

The mean annual incidence of IE for the population of children (ages 0-15y) living on the Avalon region of the province, over a five year period, from January 1, 2000 to December 31, 2004 was 107 per 100 000. This represents 74 (41 males, 33 females) newly diagnosed cases in a mean population of 13 779 over the 5-year period (www.statcan.gc.ca). The procedure utilized to calculate the incidence was as follows;

Calculation of a 5-year incidence of pediatric IE on the Avalon Peninsula:

Numerator: Total number of newly diagnosed cases seen in the pediatric neurology clinic, living on the Avalon Peninsula of the province between 2000 and 2004. The Avalon Peninsula is divided into census zones 17, 18, 19 and 20.

Denominator: 5-year averaged at-risk, age-matched population living on the Avalon Peninsula between the years 2000 and 2004.

Calculated Numerator:

$$\frac{74 \text{ new cases}}{5 \text{ year period}} = \text{mean of 14.8 new cases per year}$$

Calculated Denominator:

Mean population of children 0-15 years of age living on the Avalon Peninsula between 2000 and 2004 = 13779

$$\frac{14.8}{13\,779} = 0.00107$$

$$0.00107 \times 100\,000 = 107 \text{ per } 100\,000 \text{ children}$$

3.1.2 Clinical Data

Of the 121 patients meeting eligibility criteria, 119 cases of IE with a mean onset age of 7.3 years (range 0.25 – 15 years), were entered into the study (Figure 5). Of these 119 cases, 65 were male (55%), and 54 were female (45%). Two patients were excluded from the analysis as they were found to have an underlying disorder (neuronal ceroid lipofuscinosis) during the study.

EEG recordings were completed for all 119 patients: 86% (102) were abnormal and 14% (17) were normal. The abnormal findings were of two types; the primary idiopathic patients had generalized EEG abnormalities and the partial idiopathic patients had abnormalities consistent with benign rolandic EEG findings.

Neuroimaging (CT and MRI) was conducted in 90% (107) of patients: 97% (103) were normal and 3% (4) were abnormal. Neuroimaging was not conducted in 10% (12) of patients as they were definitively diagnosed based on EEG results. These patients had a primary generalized pattern on the EEG and imaging was not indicated. This pattern is presumed to be genetic and imaging would not be useful unless there was some other indication deeming it necessary. The abnormal neuroimaging results (4) were: mild cerebral atrophy (1), arachnoid cysts (1), cortical dysplasia (1), and benign parietal lobe lesion (1). The finding of mild cerebral atrophy was considered an incidental finding due to an EEG report strongly consistent with a generalized seizure disorder coupled with a strong family history of seizures. The arachnoid cysts (congenital lesions that occur as fluid-filled sacs between the brain and the arachnoid membrane) were also classified as an incidental finding by the attending neurologist. The findings of cortical dysplasia and a benign parietal lobe lesion led to a change in diagnoses for these patients. They were classified as having symptomatic epilepsy, not IE, based on follow-up EEGs, and therefore were excluded from the analysis.

In terms of epilepsy control, 73% (87) of patients had active epilepsy, defined by the ILAE as at least one seizure in the past five years, while 26% (31) had at least one seizure in the past year. Fifty one percent (60) are currently on an antiepileptic drug (AED) regimen.

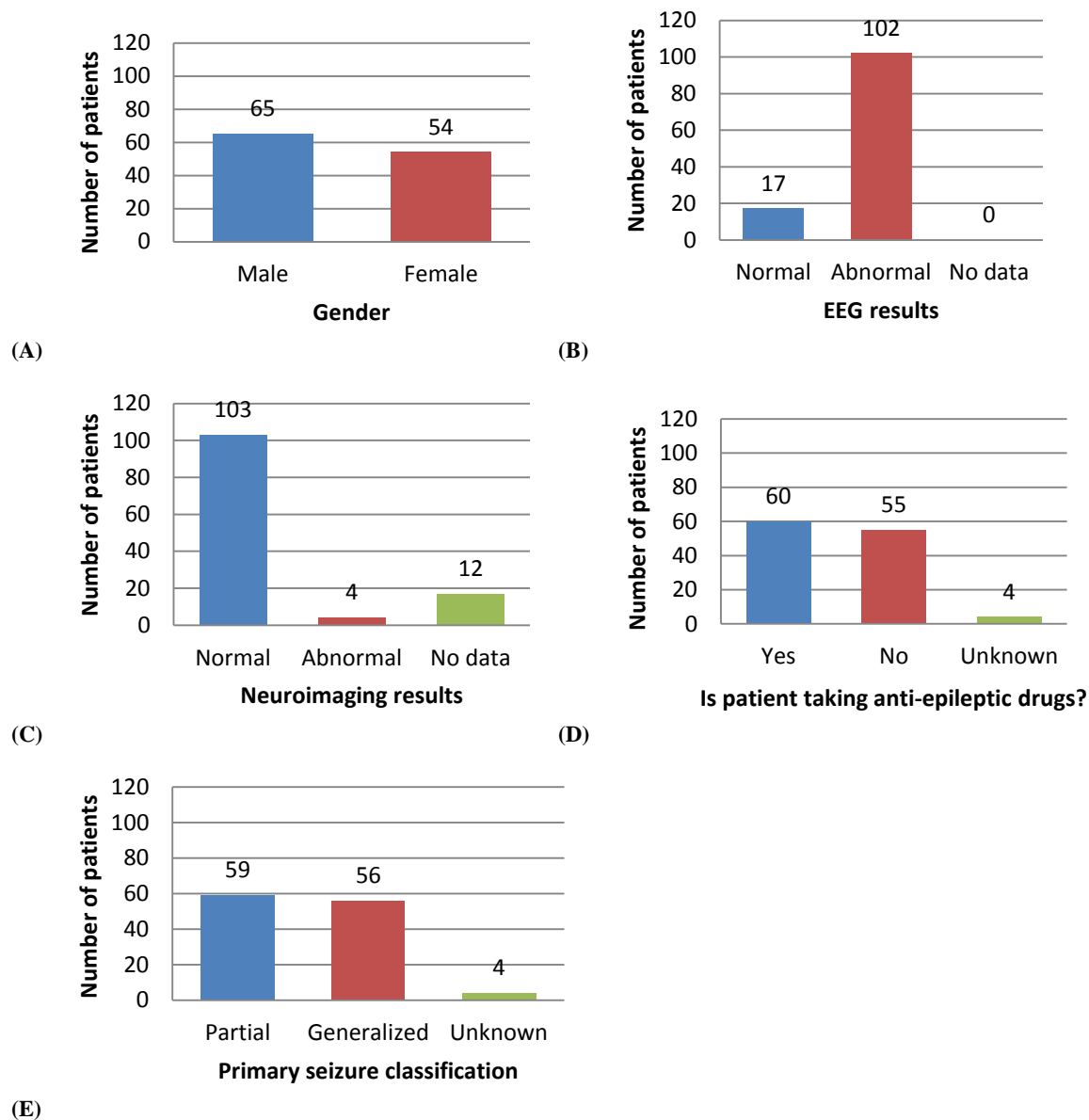


Figure 6. Bar charts depicting various clinical characteristics of the 119 IE patients.

A) Bar chart depicting number of probands as classified by gender. Males represent 55% of the total proband population.

B) Bar chart depicting number of probands as classified by EEG recording. Abnormal recordings were used by neurologists to further classify patients with a specific epileptic diagnosis.

C) Bar chart depicting number of probands as classified by neuroimaging (CT/MRI) result. Patients for which no neuroimaging data was available were definitively diagnosed by EEG based on a primary generalization pattern.

D) Bar chart depicting number of probands as classified by anti-epileptic drug (AED) status. AED status was determined at the time of last assessment.

E) Bar chart depicting number of probands as classified by primary seizure localization. When patients had multiple seizure types, they were assessed based their most common seizure classification.

3.1.3 Seizure classification and epilepsy diagnosis

Probands were classified by both primary seizure localization and epilepsy diagnosis. Fifty percent (59) of probands had partial onset seizures, 47% (56) had generalized onset seizures, and 3.4% (4) were of unknown origin. The most frequent epilepsy diagnosis was primary generalized epilepsy (PGE), not otherwise specified (18.5%), followed by childhood absence epilepsy (CAE) (16%), complex partial seizures originating from the temporal lobe (CPS-TLE) (16%), complex partial seizures originating from the parietal, frontal and occipital lobes (CPS-Other) (16%), juvenile myoclonic epilepsy (JME) (13%), benign rolandic epilepsy (BRE) (11%), epilepsy with unknown origin (6%), generalized epilepsy with febrile seizures plus (GEFS+) (2.5%), myoclonic astatic epilepsy (MAE) (1.7%), and juvenile absence epilepsy (JAE) (0.8%), respectively. Childhood absence epilepsy, juvenile myoclonic epilepsy and juvenile absence epilepsy are all major subcategories of primary generalized epilepsy. These patients were entered into separate categories to provide further diagnostic data. Those remaining in the primary generalized epilepsy category represent all other primary generalized patients, not otherwise specified (Figure 7).

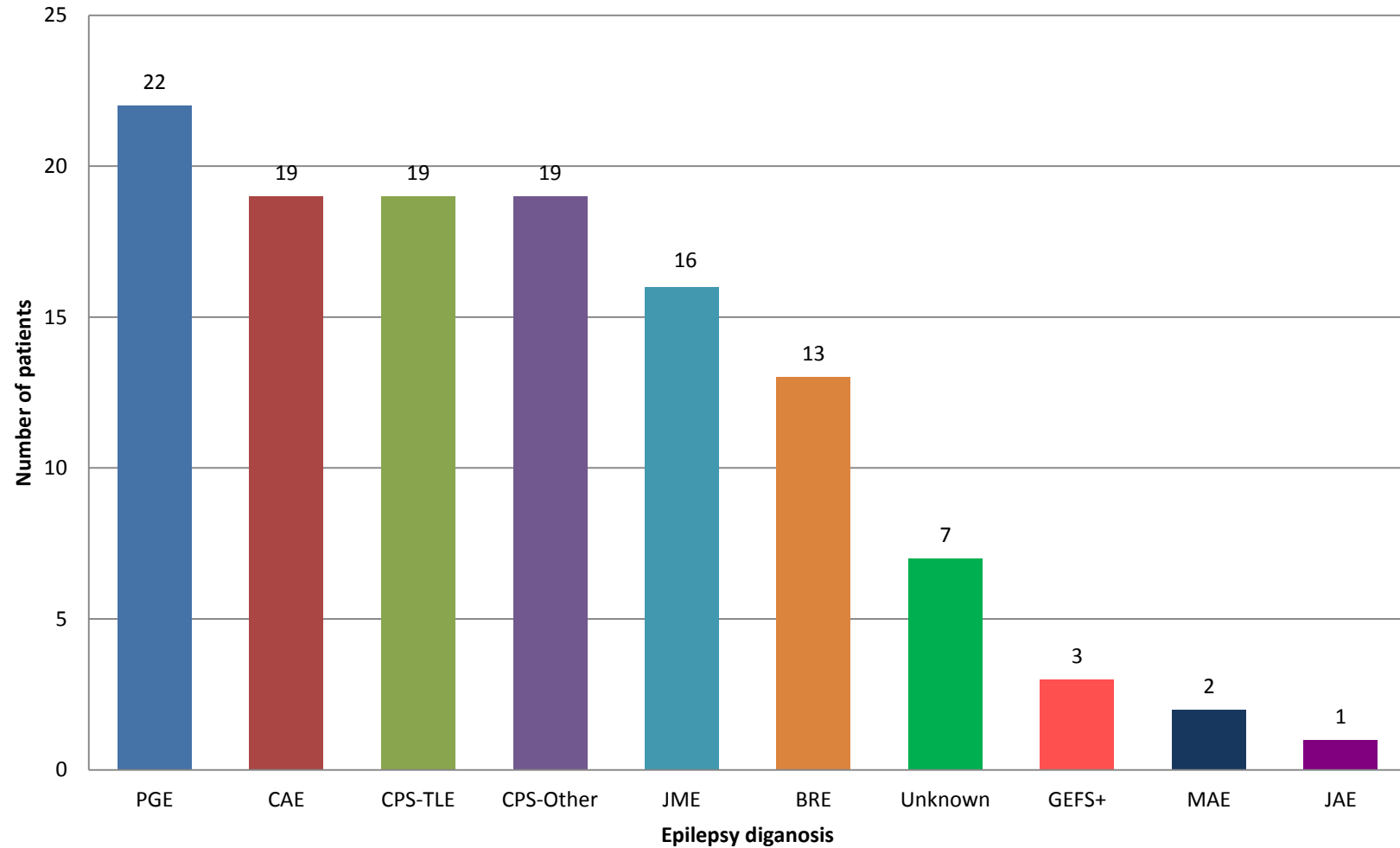


Figure 7. Bar chart depicting epilepsy diagnoses in all probands.

Key: PGE, primary generalized epilepsy, not otherwise specified; CAE, childhood absence epilepsy; CPS-TLE, complex partial seizures, temporal lobe epilepsy; CPS-Other, complex partial seizures originating from the parietal, frontal and occipital lobes; JME, juvenile myoclonic epilepsy; BRE, benign rolandic epilepsy; Unknown, origin could not be determined; GEFS+, generalized epilepsy with febrile seizures plus; MAE, myoclonic astatic epilepsy; JAE, juvenile absence epilepsy.

3.1.4 Family history data

Fifty-five (64%) of the responding probands (N=86) had a positive family history of epilepsy, defined as at least one affected first or second degree relative of the proband. Family history was also assessed in non-responders (N=31) by blind chart review and a positive family history was reported in 17 (55%) of these. Causes of epilepsy in familial disease, sporadic cases and nonresponders are given in Figure 5. Multiple causes of epilepsy occur within familial disease and sporadic cases (Table 3). Through pedigree analysis, it was determined that eight (9.3%) of the participating probands had family histories compatible with AD inheritance. Pedigrees for these eight AD families are shown in Figure 8. Clinical features of probands and clinically assessed affected family members in the eight AD families are provided in Table 3. Again, the phenotype differs widely across and within families.

Of the 55 families with a positive family history of epilepsy, 21 families provided blood samples for DNA testing to participate in the molecular phase of the study, while 34 families did not provide samples. These two groups were compared in terms of the number of affected relatives in each family. The mean number of affected relatives in the group of families participating in the molecular phase was 6.14 while the mean for the non-participating group of families was 3.9. Comparing the two means using a t statistic yielded a p value of 0.387. This suggests that the two groups are significantly different and that there may have been volunteer bias in the molecular phase of the study.

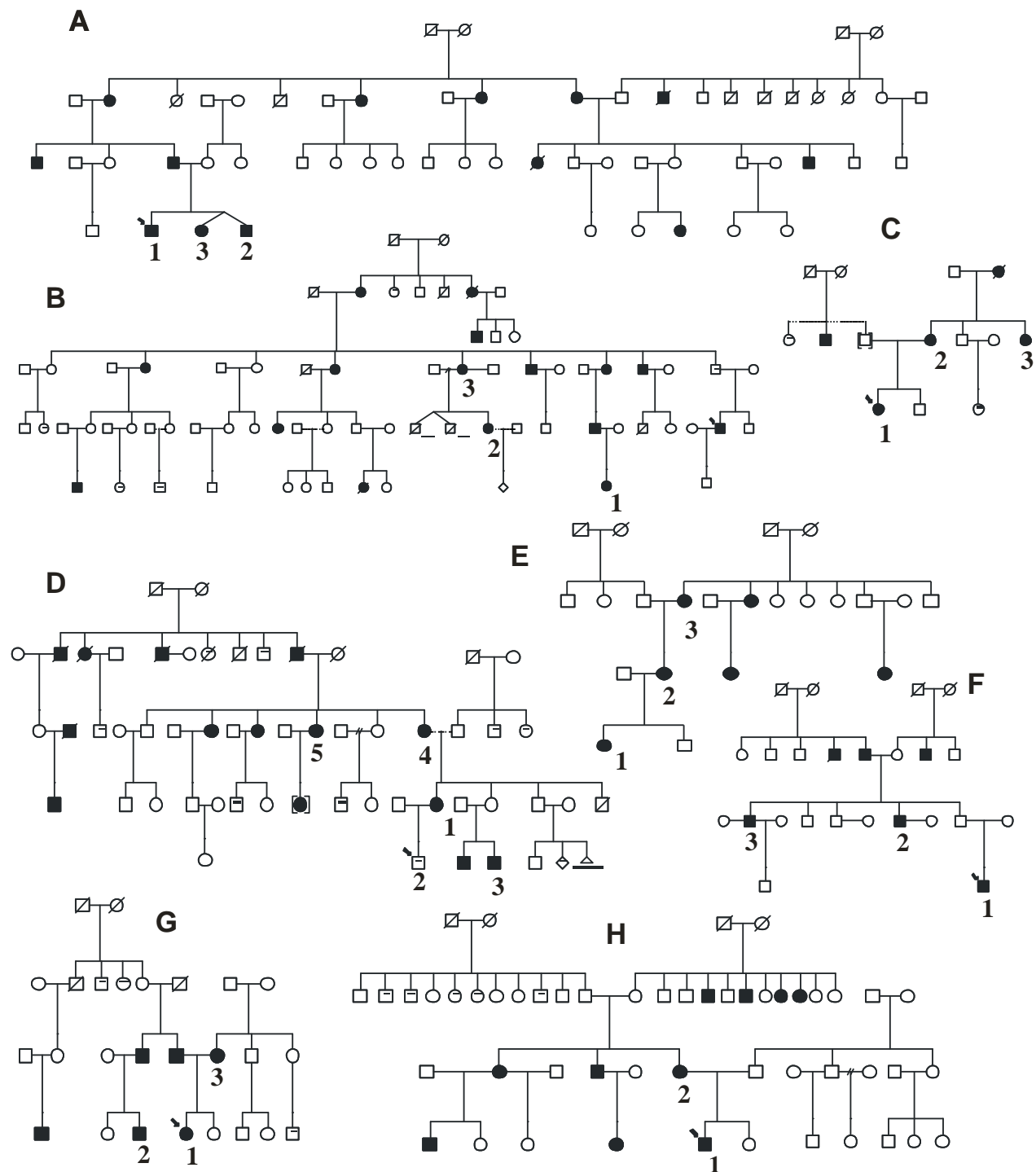


Figure 8. Pedigrees of eight families compatible with apparent AD IE. These pedigrees are reduced to include only the informative branches. Black symbols represent cases clinically affected with epilepsy, clear symbols represent no known seizure history and question marks represent cases with unknown affection status. Arrows represent the proband (index case) for each family. Letters above pedigrees identify families and numbers below each symbol identify the subjects for reference in Table 3.

Family-ID# [sex; age(y)]	Age of onset	Age of remission	Type of seizures/number	Meds	Clinical classification
A-1 [m;8]	11mo.	Cont.	FS, Ab, GTCS, SE, myoclonus/ many	Cont.	GEFS+, DD
A-2 [m;5]	1y	Cont.	FS, Ab, GTCS, SE/ many	Cont.	GEFS+
A-3 [f;5]	1y	Cont.	FS, Ab, GTCS, SE/ many	Cont.	GEFS+, DD
B-1 [m;16]	infancy	Cont.	FS, partial sz, nocturnal/ many	Stopped	CPS
B-2 [f;21]	4-5y	5y	Unknown/ 2-3	None	
B-3 [f;49]	infancy		FS/ few	None	
C-1 [f;4]	1y	3y	Partial sz (secondary generalization)/ many	Stopped	Partial to generalized
C-2 [f;39]	8y	20y	Partial sz (secondary generalization)/ many	Cont.	Partial to generalized
C-3 [f;42]	10y	~25y	Partial sz (secondary generalization)/ many	Cont.	Partial to generalized
D-1 [f;18]	16y	Cont.	GTCS/ 4	Cont.	GTCS
D-2 [m;4]	2y	Cont.	FS+/ few		FS+
D-3 [m;7]	2y	2y	Partial/ few	None	Partial epilepsy
D-4 [f;44]	14y	18y	GTCS/ few	Stopped	GTCS
D-5 [f;?]	teens	Cont.	GTCS/ many	Cont.	GTCS
E-1 [f;5]	1y	Cont.	Myoclonus/ many	Cont.	MAE
E-2 [f;35]	2-3y	5y	Unknown/ several	Stopped	
E-3 [f;64]	1y	16y	Unknown/ many	Stopped	
F-1 [f; 6]	4y	Cont.	Ab, FS, GTCS/ several	Cont.	Partial to GTCS
F-2 [m;40]	6y	12y	FS, GTCS/ 3	Stopped	GTCS
F-3 [m;51]	5y	8y	GTCS, SE/ many	Stopped	GTCS
F-4 [m;24]	2y	4y	FS/ 3	Stopped	FS+
G-1 [f;15]	2y	15y	FS, GTCS/ many	Cont.	GTCS
G-2 [m;21]	15y	17y	GTCS/ 2	None	GTCS
G-3 [f;42]	5y	31y	GTCS/ many	Stopped	Partial to GTCS
H-1 [m;8]	6y	8y	Nocturnal partial sz	Stopped	Benign Rolandic
H-2 [f;38]	Unknown	~20y	Drop attacks	Stopped	Drop attacks

Table 3. Clinical features of select patients from the eight suspected autosomal dominant pedigrees illustrated in Figure 8. Key: m, male; f, female; y, years; mo, months; Ab, absences; GTCS, generalized tonic-clonic seizures; FS, Febrile seizures; SE, status epilepticus; GSW, generalized spike wave; DS, diffuse slowing; meds, medications; DD, developmental delay; MR, mental retardation; cont., continuing.

3.1.5 *Geographic distribution*

The people of Newfoundland are distributed primarily along the coastline and, until recently, have been geographically isolated within the large bays of the province. The geographic distribution of the families and cases in this study is broadly dispersed throughout the province, closely in accordance with the distribution of the general population (Figure 9A, B & C). The distribution of families with likely autosomal dominant disease (N=8) is wide, with a family in Labrador, Central Newfoundland, the South Coast and at the South East Coast in St. Mary's Bay. A further three families live in Conception Bay and one in the city of St. John's (Figure 9B). Similar widespread geographic distribution of familial, non AD, IE (N=43) can be seen in Figure 9A. Likewise, non-familial IE patients (N=35) were randomly distributed across the province, though no such patients reside on the western half of the province (Figure 9C).

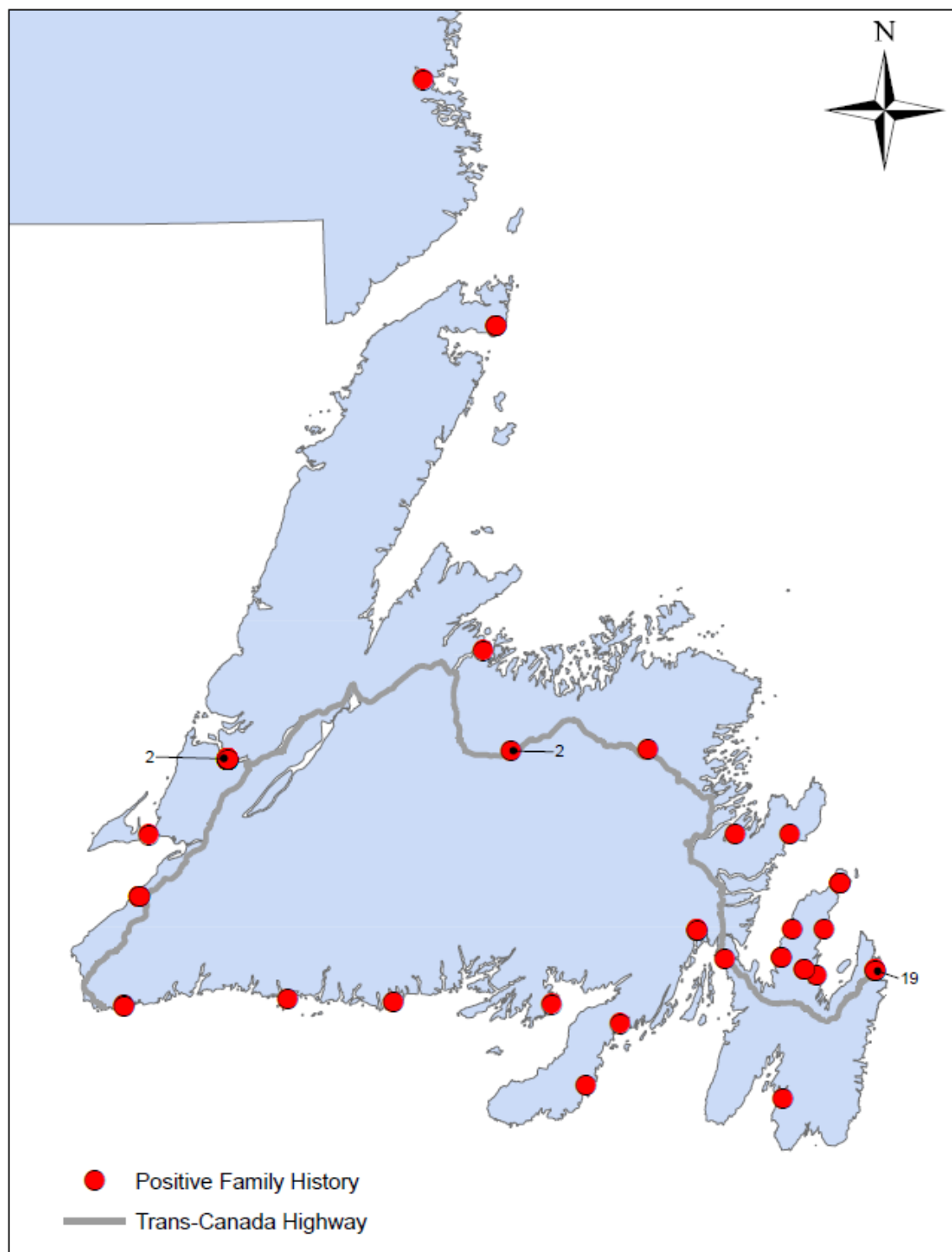
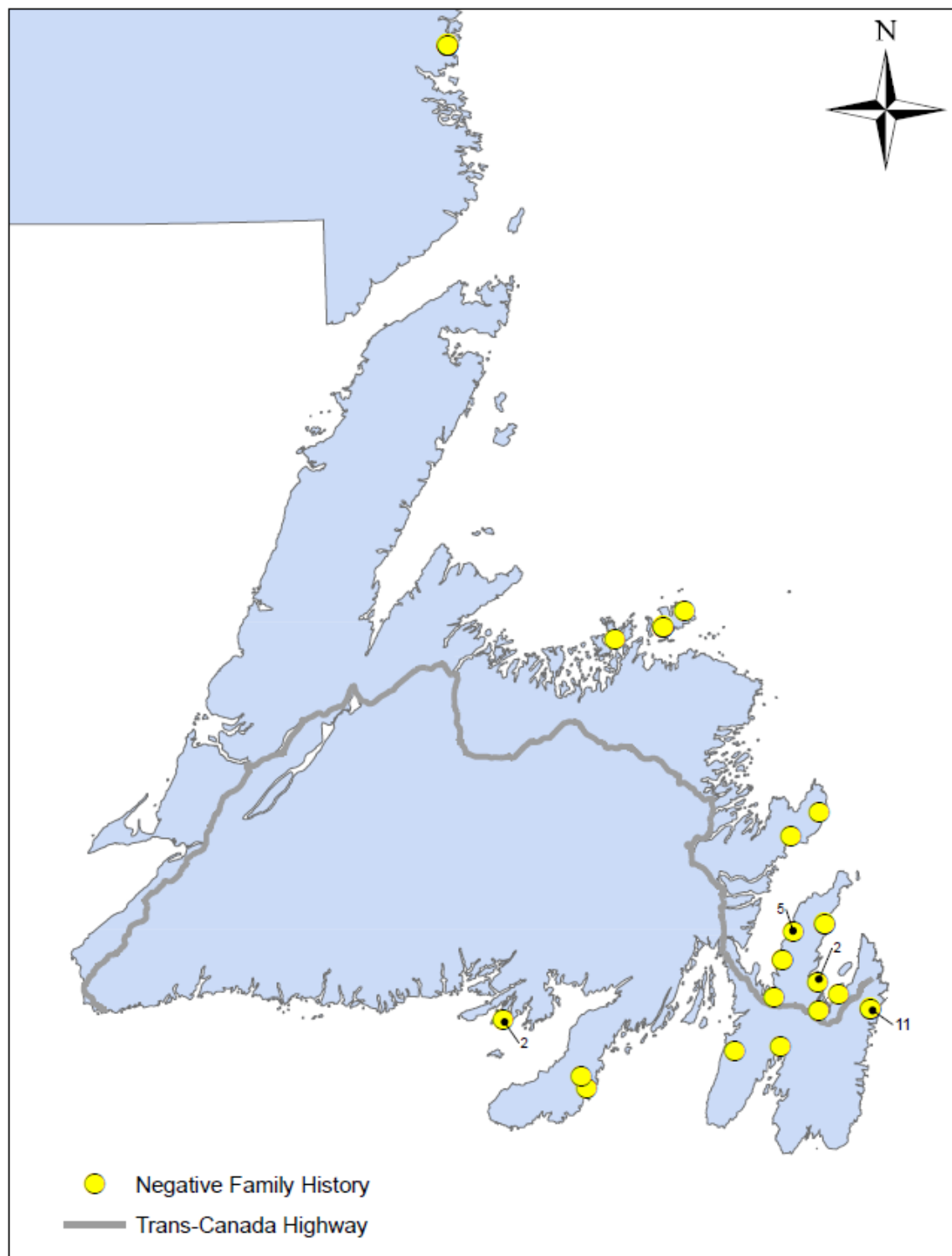


Figure 9A. Geographic distribution of pediatric IE families in the province of Newfoundland and Labrador. Numbers outside symbols indicate the number of families represented by that symbol. Cases with a positive family history are represented by red circles.



3.2 *Molecular Genetic Results*

The candidate gene selection method was employed. The strongest gene candidates were selected for sequencing based upon their previous associations with the following phenotypes seen in patients in the current study, particularly in those with a family history compatible with autosomal dominance; *SCN1A* was selected based on three patients with GEFS+ and an AD-compatible family history of disease; *SCN1B* was selected based on several patients with MAE and three patients with GEFS+ with various family history backgrounds; *SCN2A* was selected based on a few patients with probable BFNIS and three patients with GEFS+ with various family history backgrounds; *GABRA1* was selected based on several patients with both CAE and JME with various family history backgrounds; *GABRG2* was selected based on three patients with GEFS+ and an AD-compatible family history of disease; *KCNQ2* was selected based on its previous association with BFNC identified in a Newfoundland family in 1998; *KCNQ3* was selected based on its previous association with BFNC which led to the identification of *KCNQ2* in a Newfoundland family.

Upon sequencing these genes, a novel causative missense mutation in *SCN1A* (c.1162T>C; p.Tyr388His) was identified in a family with GEFS+ (Family A). Bioinformatic software programs were used to help confirm the pathogenicity of this variant. Panther determined the probability that the variant is deleterious to be 93.9%. Polyphen predicted the variant to be “probably damaging”. SIFT predicted the amino acid change to be “deleterious”.

No pathogenic variants in *SCN1A*, *SCN2A*, *SCN1B*, *KCNQ2*, *GABRA1*, *GABRG2* or *KCNQ3* were found in any of the remaining 20 families, which included six with presumed AD inheritance. All variants with nomenclature and bioinformatic analyses are listed below in Table 4.

Gene	Exon/ Intron	Variant nomenclature	rs number	Predicted protein change	SIFT	PolyPhen	Allele freq	CAU MAF
<i>SCN1A</i>	Intron 1	c.265-83A>T	NA	NA non-coding	NA	NA	0.07 (3/42)	NA
	Intron 6	c.965-21C>T	NA	NA non-coding	NA	NA	0.07 (3/42)	NA
	Exon 8	c.1162T>C	NA	p.Tyr388His non- synonymous	deleterious	probably damaging	0.02 (1/42)	NA
	Exon 9	c.1212A>G	rs 7580482	p.= (Val404Val) synonymous	tolerated	benign	0.47 (20/42)	0.42
	Exon 13	c.2167T>C	NA	p.= (Val723Val) synonymous	tolerated	benign	0.02 (1/42)	NA
	Exon 16	c.3310G>A	NA	p.Alal104Thr non- synonymous	tolerated	benign	0.02 (1/42)	NA
	Exon 17	c.3472G>A	NA	p.Glu1158Lys non- synonymous	tolerated	benign	0.02 (1/42)	NA
<i>SCN1B</i>	Intron 1	c.40+15G>T	rs 72556351	NA non-coding	NA	NA	0.07 (3/42)	0.15
	Intron 4	c.590+68G>A	rs1467190 86	NA non-coding	NA	NA	0.02 (1/42)	0.04
	Exon 6	c.*86A>C	rs 2278996	NA non-coding	NA	NA	0.10 (4/42)	0.14
<i>SCN2A</i>	Intron 2	c.-51-16C>T	rs 75551103	NA non-coding	NA	NA	0.02 (1/42)	NA
	Intron 17	c.3400-71C>T	rs 3769951	NA non-coding	NA	NA	0.36 (15/42)	0.30
	Intron 13	c.2017-89A>G	rs 74598858	NA non-coding	NA	NA	0.05 (2/42)	0.07
	Exon 23	c.4287T>C	rs1502099 84	p.= (Tyr1429Tyr) synonymous	tolerated	benign	0.02 (1/42)	NA
<i>GABRA1</i>	Intron 7	c.1059+22G> A	rs1839738 84	NA non-coding	NA	NA	0.02 (1/42)	NA
<i>KCNQ2</i>	Intron 1	c.297-170C>T	rs2297387	NA non-coding	NA	NA	0.36 (15/42)	0.38
<i>GABRG2</i>	Exon 5	c.588C>T	rs211037	p.= (Asp196Asp) synonymous	tolerated	benign	0.33 (14/42)	0.39
<i>KCNQ3</i>	Intron 5	c.573+25T>C	rs 17575971	NA non-coding	NA	NA	0.02 (1/42)	0.08

Table 4. List of sequencing variants identified in functional candidate genes sequenced in IE patients. Allele freq, refers to allele frequency found in study samples; CAU MAF, minor allele frequency in Caucasian population (as reported in 1000 Genomes and/or Ensembl Genome Browser).

3.3 Results of Family A Study

3.3.1 Clinical Features of Family A

In the current study, we report a four generation family with autosomal dominant GEFS+ with a novel mutation in *SCN1A*, which extends the phenotypic spectrum associated with GEFS+ to include neuropsychiatric issues; ataxia and social anxiety. The founders of this Newfoundland family originate from County Cork, Ireland. The complete pedigree consists of 97 relatives across 5 generations segregating an autosomal dominant form of epilepsy. The core pedigree depicts only the affected branches in this family, which includes 11 individuals diagnosed with epilepsy in three successive generations (Figure 10). Marked clinical heterogeneity between affected relatives with a variety of seizure types and neurological deficits was observed through review of medical records and EEGs, and also through seizure questionnaires and home visits. All clinically affected relatives (n=11) had seizure disorders starting in childhood with a mean onset age of 2.1 years (range 0.75 – 5.5 years) (Table 5). Of these, nine (IV:1, IV:2, IV:3, III:3, III:2, II:7, II:9, III:12 and III:16) had generalized tonic-clonic seizures (GTCS) which occurred with and without fever, and two (II:2 and IV:5) had febrile seizures only. Regarding seizure presentation, five (IV:1, IV:2, IV:3, II:7 and III:12) had multiple episodes of status epilepticus, four (IV:1, IV:2, IV:3 and III:12) had absence seizures, one (IV:1) had myoclonic seizures, and one (IV:5) had atonic seizures. Other neuropsychiatric disorders were also prominent, with five (IV:1, IV:2, III:2, III:12 and III:16) individuals presenting intellectual disabilities, three (II:7, II:9 and III:16) with debilitating psychiatric disease, and two, including the proband (IV:1, III:12), with ataxia. None of the eight spouses had any personal or family history of seizures.

The proband (IV:1, Figure 10) had his first seizure (afebrile GTCS) at 11 months of age. He began having myoclonic seizures at two years and by three years of age was experiencing approximately 10 myoclonic seizures per day. These seizures were controlled with Divalproic

Acid and Ethosuximide to a rate of one per 2-3 days. In addition, he had frequent generalized tonic-clonic (GTC) and absence seizures despite treatment with Valproic Acid and Ethosuximide, as well as rescue Benzodiazepines. He has had two episodes of status epilepticus. CT head scan at three years of age was normal. EEG at three years of age showed generalized epileptiform discharges, slowing, and polyspike and wave discharges. EEGs at four, six and seven years of age were normal. Of particular note are his neurologic signs. At eight years old he displayed moderate global developmental delay, ataxia and behavioral concerns. He repeated kindergarten and receives special education support at school (Criteria C*), but has had no developmental regression. He continues to have GTCS approximately once every three to four months and remains on Valproic Acid. His most recent episode of status epilepticus was four years ago, at the age of four years.

The sister of the proband (IV:2) was born at 34+3 weeks gestation (twin A). Her first seizure, a febrile GTCS, occurred at the age of one year two weeks. She has since had many GTC and absence seizures, and has had seven hospital admissions for episodes of status epilepticus, lasting 30 to 80 minutes. EEGs (at one, two and three years of age) and a CT head scan (at two years of age) were normal. She is now five years old, has mild developmental delay and GTC and absence seizures (persisting at a rate of approximately one per month) on treatment with Topiramate and Valproic Acid. Kindergarten entry was delayed by one year due to developmental delay.

The brother of the proband (IV:3, twin B) experienced his first seizure at the age of one year two weeks. This was an afebrile partial seizure progressing to a secondary generalized tonic-clonic seizure. He has since had both febrile and afebrile onset generalized seizures. He

* *Criteria C: Special education support provided to children exhibiting moderate global/severe/profound cognitive delay.*

has had two episodes of status epilepticus. EEGs (at two and three years of age) and a CT head scan (at one year of age) were normal. He is now five years old, shows normal cognitive development and his seizures persist at a rate of approximately one per three to six months, despite treatment with Valproic Acid. There have been no reported learning or behavioral issues.

Individual III-14 was considered to be the only obligate carrier in this family as she had an affected parent and child. She had no history of seizures which was confirmed by medical chart review. However, through interview and medical chart review, it was determined that she had neuropsychiatric symptoms including anxiety and depression.

Of the remaining eight affected individuals, all except two have developed afebrile seizures requiring antiepileptic medication. Three of these individuals with afebrile seizures have developmental delay, all of whom have a history of multiple seizures refractory to medication.

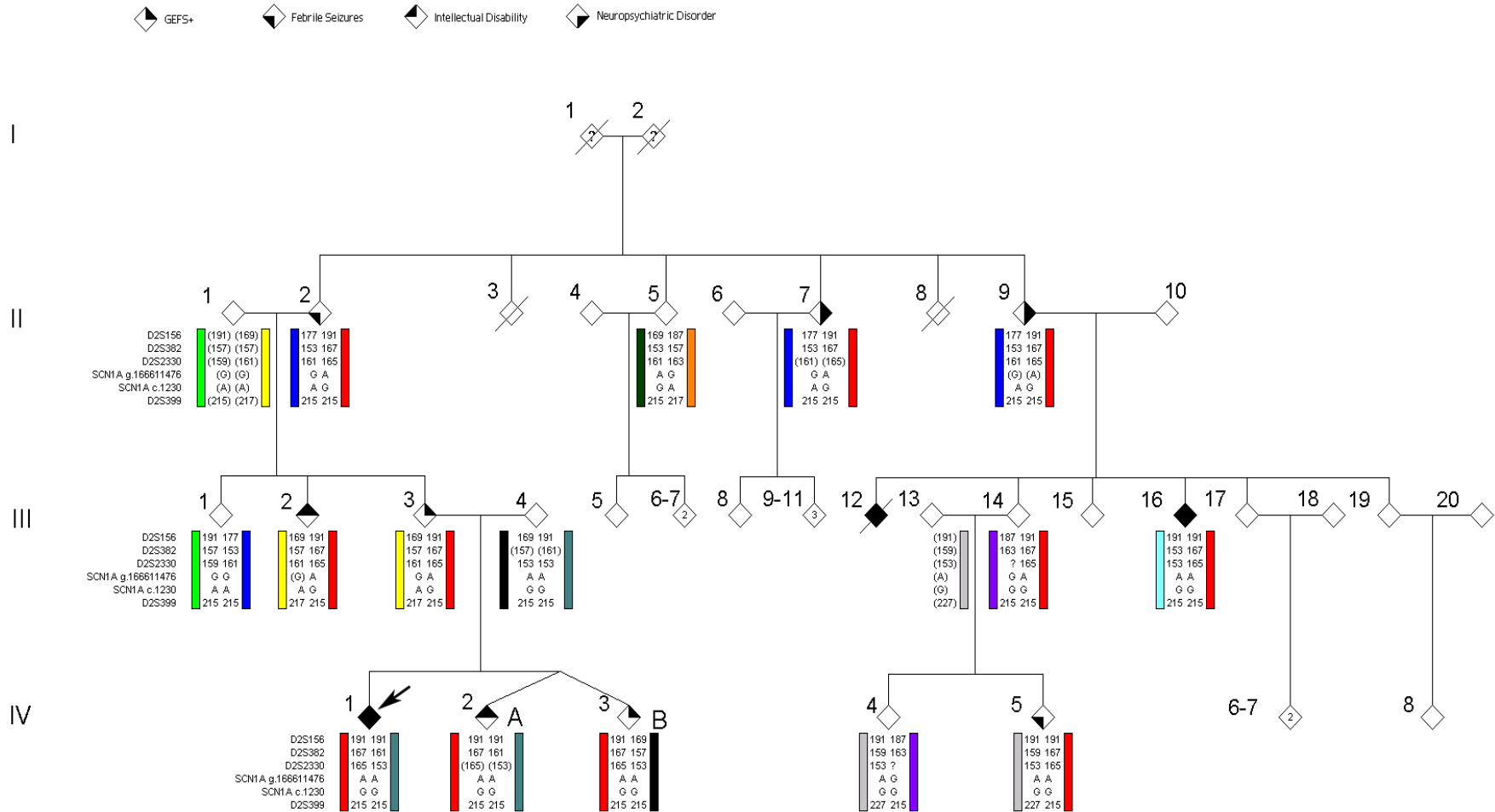


Figure 10. Core pedigree of Newfoundland family showing both typical and novel features of GEFS+ associated with the epilepsy-associated haplotype (red) on chromosome 2q24 in the vicinity of *SCN1A*. Markers are listed on the left and alleles given in size (base pairs) or specific nucleotide. The proband is indicated by an arrow. Gender has been masked using diamond symbols to protect confidentiality.

Pedigree reference [sex; age]	Age of onset/ remission	Type of seizures/ number	EEG	Neuroimaging (MRI/CT)	AEDs	Intellectual disability	Psychiatric/ other neurological disorders	Clinical classification of epilepsy
IV:1 [m; 8y]	11 mo./ cont.	Ab, FS, GTCS, SE, myoclonus/ >100	GSW	MRI- normal CT-normal	Cont.	Moderate DD	Ataxia	GEFS+
IV:2 [f; 5y]	1 y/ cont.	FS, Ab, GTCS, SE/ >50	Bilateral epileptiform discharges in frontal region	CT-normal	Cont.	Mild DD	None	GEFS+
IV:3 [m; 5y]	1 y/ cont.	FS, Ab, GTCS, SE/ >50	Normal	CT-normal	Cont.	None	None	GEFS+
III:3 [m; 32y]	1 y/ 29 y	FS, GTCS/ many	NA	CT-normal	Cont.	None	None	GEFS+
III:2 [m; 34y]	1 y/ 15 y	FS, GTCS/ 5-10	Normal	CT-normal	Stopped	Learning difficulty, probable ADHD	None	GEFS+
II:2 [f; 57y]	5-6 y/ teens	FS/ 2	NA	NA	None	None	None	FS
II:7 [f; 63y]	Infancy/ 20-22 y	GTCS, SE/ >50	NA	NA	Stopped	None	Anxiety/ Depression ^a	GTCS
II:9 [f; 61y]	4-5 y/ 23 y	FS, GTCS/ 4	NA	NA	Stopped	None	Social anxiety/ depression	GEFS+
III:12 [f; deceased 27y] DNA not available	9 mo./ 27 y	Ab, FS, GTCS, SE/ 100s	GSW, Rhythmic and paroxysmal slowing	CT-normal	Cont.	Severe MR	Ataxia	GTCS Cause of death: multiple seizures
III:16 [m; 41y]	5 y/ 20 y	FS, partial to GTCS/ many	Normal	CT-normal	Cont.	Mild DD	Social anxiety ^b	GEFS+
IV:5 [f; 4y]	16 mo./ 22 mo.	FS, atonic/ 2	Normal	NA	None	None	None	2 atonic febrile seizures
III:14 [f; 35y]	18y/ cont.	None	NA	NA	None	None	Anxiety/ Depression	None

Table 5. Clinical features of affected members of Family A.

Key: m, male; f, female; y, years; mo, months; Ab, absence; GTCS, generalized tonic-clonic seizures; FS, Febrile seizures; SE, status epilepticus; GSW, generalized spike wave; AEDs, Anti-epileptic drugs; DD, developmental delay; MR, mental retardation; ADHD, Attention Deficit Hyperactivity Disorder; cont., continuing. ^aPatient was treated in a psychiatric unit. ^bPatient lives with his parents and is unable to work due to social anxiety.

3.3.2 Genetic Study of Family A

Genotyping data from 16 members of Family A (10 affected, six unaffected) consisted of base pair repeat sizes for four microsatellite markers (D2S2330, D2S156, D2S399 and D2S382) and allele calls for two intragenic SNPs spanning the *SCN1A* gene on chromosome 2q24. Haplotypes were constructed for all 16 family members according to Mendelian inheritance rules. Visual analysis of the haplotypes in this pedigree confirmed co-segregation of epilepsy with a haplotype on 2q24 (Figure 10). Direct sequencing of *SCN1A* in 16 DNA samples from this family (10 affected, six unaffected) revealed 7 sequence variants that co-segregated on the epilepsy-associated haplotype (Table 6). However, only one of these, a c.1162T>C transversion in exon 8 of *SCN1A* (Figure 12), was found in all clinically affected relatives, predicted an amino acid substitution in the first domain of the sodium ion channel protein (NaV1.1), and was not seen in any population controls (Table 6). This missense mutation predicts a deleterious transition from a highly conserved (Figure 12A) polar neutral residue (Tyrosine) to a polar basic residue (Histidine) within the pore region of domain one of the sodium channel protein (Figure 12B). Bioinformatics software tools provided supportive evidence that this missense mutation is pathogenic and segregation analysis of the pedigree confirmed that it is also highly penetrant.

A Simplified method for Segregation Analysis (SISA) was used to determine the penetrance and expression of the variant in this family (Moller, Clark et al. 2010). It was determined that the probability that cosegregation between the disease phenotype and the genetic variant occurs by chance is 0.2%. Based on expressivity of the mutation within these 16 members, penetrance was 91%. Specifically, all 10 affected family members carried the mutation and 5 of the 6 unaffected members did not carry the mutation. The only unaffected mutation carrier (III-14) was deemed an obligate carrier due to the fact that she has an affected

child. This low probability of cosegregation by chance and high penetrance indicate that the genetic variant has a high likelihood of causing the phenotype and is considered to be an autosomally, dominantly inherited trait.

Exon/Intron	Variant Nomenclature	Classification	Amino Acid Change	Segregation of Alleles/ Verification
Intron 1	c.265-83A>T	Noncoding	No	Does not predict a splice site^a
Intron 6	c.965-21C>T	Noncoding	No	Does not predict a splice site^a
Exon 8	c.1162T>C p.Y388H	Missense	p.Tyr388His	Heterozygous in all affecteds Not present in 190 control alleles
Exon 9	c.1212A>G p.V404V rs7580482	Synonymous	p.= (Val404Val)	Minor allele frequency = 42%^b
Exon 13	c.2167T>C	Synonymous	p.= (Val723Val)	Not present in all affecteds
Exon 16	c.3310G>A	Missense	p.Ala1104Thr	Not present in all affecteds
Exon 17	c.3472G>A	Missense	p.Glu1158Lys	Not present in all affecteds

Table 6. Sequencing variants identified in *SCN1A* (NM_006920) in clinically affected subjects.

^aBDGP: Splice Site Prediction by Neural Network was used to determine if variants predict splice sites. ^bNCBI dbSNP was used to determine the allele frequency of previously reported variants.

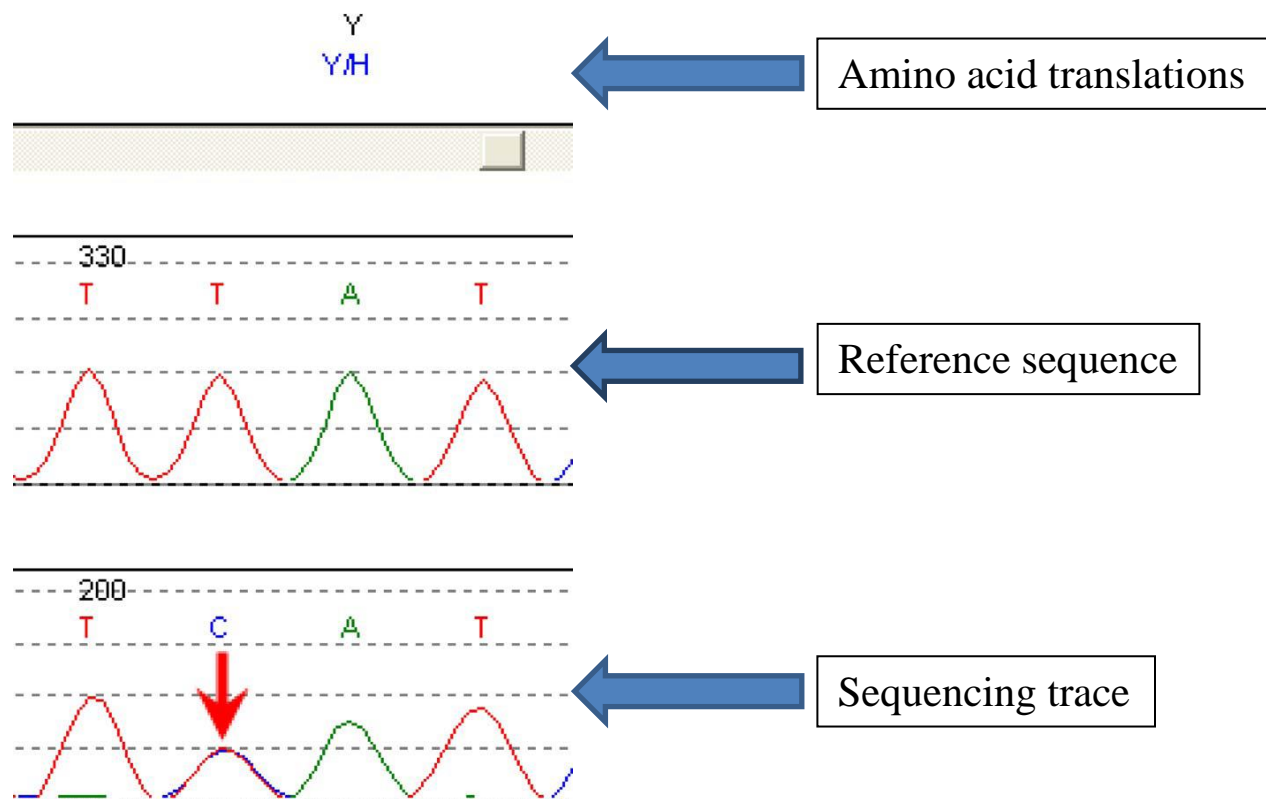


Figure 11. Sequencing traces of exon 8 of *SCN1A* (only forward direction shown) showing the c.1162T>C transition of the proband's (IV:1, Figure 10) genomic DNA aligned with the reference sequence. The amino acid translations (top) show the p.Y388H amino acid substitution.

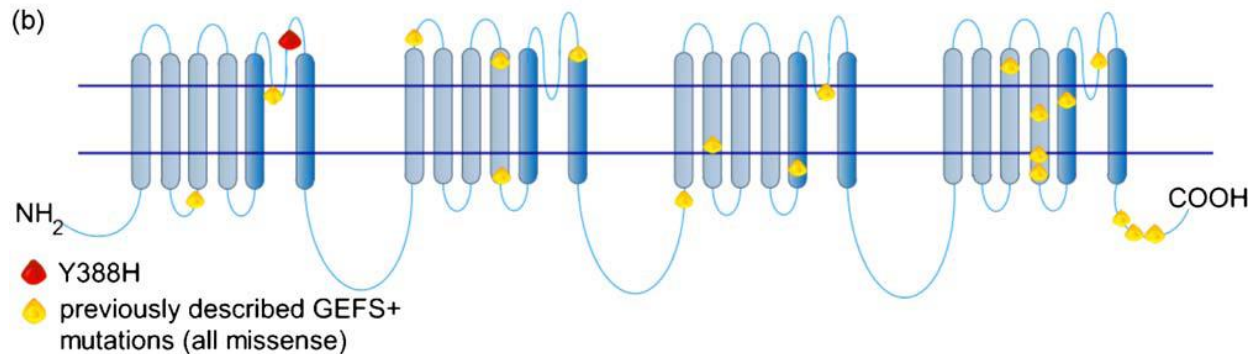
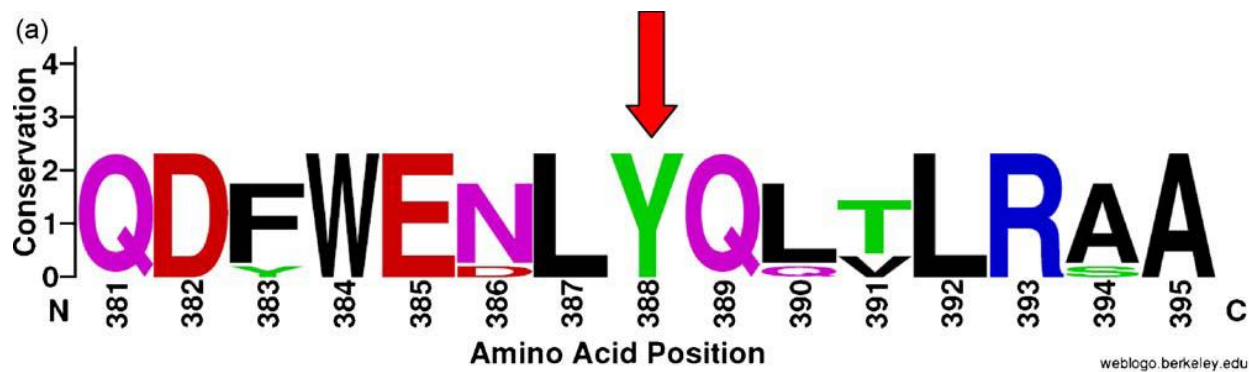


Figure 12. Conservation of *SCN1A* in the vicinity of Y388 and location of p.Y388H.

(A) Weblogo was used to align orthologs from *Homo sapiens* (NP_008851), *Pan troglodytes* (XP_515872), *Mus musculus* (NP_061203), *Rattus norvegicus* (NP_110502), *Danio rerio* (NP_956426), *Drosophila melanogaster* (NP_001036280), and *Nasonia vitripennis* (NP_001128389). The tyrosine (Y) residue at position 388 of the *SCN1A* gene across sodium channel genes is marked by a red arrow.

(B) Y388H (red triangle) results in the substitution of a neutral polar tyrosine residue to a polar basic histidine in the loop between the S5 and S6 segments of domain 1 of the *SCN1A* protein. Segments 5 and 6 (darker blue) form the ion channel pore and segment 4 is the voltage sensor.

Chapter 4

Discussion

4 Discussion

4.1 Summary

This study characterizes pediatric IE in the context of a historically isolated founder population on the island of Newfoundland. This provincial population-based study endeavoured to describe the incidence, genetic epidemiology, and genetic etiology of pediatric IE. The mean annual incidence of IE was discovered to be three times higher than reported rates in comparison populations. The scattered geographic distribution of familial and non-familial cases of IE in different isolates throughout the province suggests that the disease is genetically heterogeneous. The high clinical variability within and between families lends further evidence to this finding. A novel mutation in the *SCN1A* gene was identified and the associated phenotype was found to have some novel features as well. These findings have been published in *Seizure* (2009) and *Epilepsy Research* (2011).

4.2 Study design: Translational research

The current study demonstrates the utility of obtaining thorough and accurate clinical data to fuel laboratory research. Consequently, laboratory findings were generated that aided the clinical care of patients. This “bedside-to-bench-to-bedside” approach demonstrates the importance of all facets of research interacting to produce patient-tailored care. I obtained comprehensive clinical data from all available patients and family members through multiple clinical assessments, interviews, questionnaires, medical chart reviews, EEGs, and neuroimaging. Using this plethora of data, each patient had the best possible epilepsy classification. This data was also used for hypothesis generation. I generated a list of candidate genes, genotyped and/or DNA sequenced patients at these loci, and determined if any pathogenic

variants were present. In the case of Family A, identifying the pathogenic mutation in *SCN1A* had an immediate clinical impact on patient care. The results of genetic testing were relayed to the family via genetic counseling. Subsequently, several other relatives were also contacted and clinically assessed. The family is now receiving clinical follow-up including genetic counseling. Further, an anti-epileptic drug that several family members were taking prior to this study is known to exacerbate sodium channel disorders. Thus, this finding informed better pharmacologic management for affected family members through the use of a more appropriate and effective AED regimen and the patients have experienced more favorable seizure outcomes as a result.

4.2.1 Extensive patient data ascertainment

The large sibship sizes and close involvement with the provincial neurology program enabled collection of extensive family history data, and construction of at least three-generation pedigrees for each family. The pedigree data in this study is more comprehensive than most other epidemiological studies of epilepsy which have obtained brief family histories from chart review and have not reported pedigrees (Sidenvall, Forsgren et al. 1993; Sidenvall, Forsgren et al. 1996; Beilmann, Napa et al. 1999; Freitag, May et al. 2001; Kwong, Chak et al. 2001; Mullins, O'Sullivan S et al. 2007). The relatively high proportion of families with epilepsy suggestive of a mutation in a single gene causing disease may be partly due to the rigorous collection and analysis of pedigree data. While this may present challenges in drawing comparisons between populations, and limits the generalizability of familial rates in NL to other populations, it also allows a more accurate assessment of the genetic contribution to IE than previous studies.

4.3 *Epidemiology*

This study demonstrates that the incidence of pediatric IE in the Avalon Region of Newfoundland and Labrador is approximately three-fold higher than the average incidence reported in other developed countries (Kotsopoulos, van Merode et al. 2002). The proportion of IE with familial disease is high (55/86, 64%) as compared to a study in the Irish population which found that 48% of IE patients had an affected first or second degree relative (Mullins, O'Sullivan S et al. 2007). This was the only other published study to provide data on the proportion with familial IE. The familial rate is high in both responders (64%) and non-responders (55%), indicating that the familial rate was not dramatically affected by response bias. The proportion of pedigrees suggestive of AD inheritance (8/86, 9.3%) is also high; most population-based studies report a few rare cases of AD families with only 1% of IE being attributed to a monogenic cause (Gardiner 2000; Robinson and Gardiner 2004). The incidence of IE on the Avalon Peninsula of the province is high and the genetic contribution to the etiology of IE, across the province, is also high as compared to population-based studies in other developed regions (Kotsopoulos, van Merode et al. 2002; Mullins, O'Sullivan S et al. 2007).

4.4 *Geographic Distribution / Founder Effects*

The geographic locations of study subjects/families were mapped to identify patterns and look for trends that may be present. This information is important for multiple reasons. A clustering of cases in a geographical region is highly relevant to genetic studies as it may indicate a single mutation (perhaps a founder mutation) is causing the disease in multiple cases/families. This would likely be the result of a common ancestor that may or may not be known to the

families. A clustering of cases in a given region could also be attributed to environmental factors. For example, it could be hypothesized that a given exposure could increase the rate of *de novo* mutations causing epilepsy. Also, geographic distribution of disease is important to health care providers and policy makers as it can aid in determining the distribution of health services based on the needs of each given area.

The geographic distribution of both the cases and families in this study, shows little clustering. Families with likely AD disease or familial non-AD disease are dispersed widely throughout the province in a manner comparable to the distribution of the general population. Despite the high heritability quotient in this population, and the demonstration of founder effects in inherited colorectal cancer (Stuckless, Parfrey et al. 2007) and arrhythmogenic right ventricular cardiomyopathy type 5 (Merner, Hodgkinson et al. 2008), founder effects likely cannot explain the genetic contribution of epilepsy in its entirety. Although Newfoundland has long been regarded as an isolated founder population (Bear, Nemec et al. 1987; Bear, Nemec et al. 1988), it has been noted in a study of genome-wide distribution of linkage disequilibrium (LD) that the Newfoundland population has relatively low LD as compared to 10 other well-described population isolates (Service, DeYoung et al. 2006). Newfoundland is most likely not a single genetic isolate but rather a series of isolates scattered around the coast of the island. Thus, it may be more accurate to conclude that the high incidence of IE and familial disease is due to the rigorous ascertainment methods more so than founder effects. The geographic distribution is similar to that seen in Bardet-Biedl Syndrome and Lynch Syndrome, both monogenic conditions associated with multiple genetic founders, and caused by mutations in multiple different genes, which differ across genetic isolates (Webb, Dicks et al. 2009).

There are several methodological advantages to studying a genetically isolated population, such as that of Newfoundland. The province's founder population is characterized by genetic homogeneity which yields a high signal to noise ratio for discerning etiology. In addition, more complete ascertainment and accurate phenotypic information can be obtained from typical Newfoundland families which tend to be large, cohesive, and have extensive knowledge of their family history. Clinical and genetic investigation of these families will provide information pertaining to the phenotypes and genotypes of the families as well as the modes of inheritance and clinical expression of IE in the entire Newfoundland and Labrador population.

As genes have been identified in several autosomal dominant epilepsy families in other populations, it is likely that families with Mendelian inherited epilepsy disorders are present in the Newfoundland and Labrador population. Further clinical and genetic investigation of these families will provide more information pertaining to the phenotypes and genotypes of the families as well as the modes of inheritance and clinical expression of IE in the entire Newfoundland and Labrador population.

4.5 *Summary of Family A Study*

This study reports a comprehensive clinical study of Family A, a family with an autosomal dominant history of extended spectrum GEFS+ and a novel missense mutation in *SCN1A*. The core pedigree of this large multiplex family had 11 individuals with a history of seizures over three generations. A broad spectrum of epilepsy phenotypes was seen across family members, with most having forms of generalized epilepsy such as FS+, absences and GTCS. The phenotype prevalent throughout this family is compatible with the GEFS+ spectrum

(Scheffer and Berkovic 1997) and the inheritance pattern in this pedigree is compatible with an autosomal dominant mode of inheritance. It was hypothesized that an AD mutation was segregating with the seizure disorder prevalent in this family. Assuming AD inheritance, there was one obligate carrier in which the lack of a seizure history could be explained by incomplete penetrance, subclinical expression of features (e.g. unreported nocturnal seizures), unknown environmental protective factors, or other complex protective genetic variations yet to be identified. All living family members with a history of seizures have a heterozygous T>C transition at nucleotide 1162 of *SCN1A* which results in a tyrosine to histidine substitution (p.Y388H).

The clinical findings in this study expand the phenotype associated with GEFS+ to include a range of other neuropsychiatric deficits, including mood disorders and ataxia, in addition to familial epilepsy. There have been no previous reports of familial ataxia in families with GEFS+ as the primary presentation. In this family, ataxia was observed in two clinically affected family members (IV:1 and III:12), which has been previously reported in association with mutations in the pore-forming regions of *SCN1A* in those with an SMEI phenotype (Kanai, Hirose et al. 2004). In addition, there are three affected mutation carriers that presented with debilitating psychiatric disease (anxiety and depression) in addition to epilepsy. Although not clinically examined with neuropsychological scales through the scope of this study, medical records suggest that these mutation carriers present an expanded GEFS+ phenotype including a range of other neuropsychiatric deficits.

Unfortunately, the occurrence of mood disorders such as depression and anxiety are relatively common in the general population, making it difficult to prove that these symptoms represent a novel extension of the current GEFS+ phenotype. Alternatively, ataxia, being much

less common in the general population and previously reported in SMEI patients, is easier to accept as a novel phenotypic extension. Identifying additional GEFS+ individuals and families with these symptoms will give credence for them being included in this syndrome. Additionally, once the genetic predispositions for GEFS+ are identified, it may be possible to ascertain specific genotype-phenotype relationships between particular mutated genes and/or mutations in mood disorders and ataxia. However, it is also possible that the observations made between GEFS+ and the extended phenotype occur by chance and that they just happen to be present in some mutation positive family members. A good example of the importance of deep phenotyping in many families with a particular condition is Bardet-Biedl syndrome (BBS). BBS is a rare autosomal recessive multi-system disorder which is more prevalent in Newfoundland (Moore, Green et al. 2005). The cardinal manifestations were originally thought to be retinal dystrophy, obesity, mental retardation, polydactyly and hypogenitalism in males. However, with additional studies, particularly from the Newfoundland group (O'Dea, Parfrey et al. 1996), renal malformation was identified as a very important cause of morbidity and mortality and was subsequently added as a primary criteria. Additional studies from around the world have also added many other secondary characteristics to the BBS phenotype including neurological, speech, and language deficits, behavioural traits, facial dysmorphism, and dental anomalies (Beales et al., 1999). What was once a very rare and relatively unknown phenotype became a widely investigated syndrome and a very genetically heterogeneous disease. The breadth of the phenotype was not known until decades after this syndrome was intensely investigated during the genetics and genomics era.

Interestingly, mutations in *SCN1A* have been previously reported to be associated with other neuropsychiatric disorders of similar pathology. Panic disorder and Asperger syndrome

were present in two individuals in a GEFS+ family with a missense mutation in *SCN1A*, and variants in the *SCN1A* and *SCN2A* genes were observed in six patients with autism (Weiss, Escayg et al. 2003; Osaka, Ogiwara et al. 2007). Heterozygotes for a null allele of another sodium channel gene, *SCN8A*, showed a variety of cognitive defects, including mental retardation and behavior disorders (Trudeau, Dalton et al. 2006). Additionally, a novel missense mutation in *SCN1A* was identified in a family with familial hemiplegic migraine, widening the phenotype associated with mutations in this gene (Gargus and Tournay 2007).

The majority of affected individuals in this family present a more severe phenotype than is usual with GEFS+. Of the ten affected individuals known to have the *SCN1A* mutation, four have developmental delay (IV:1, IV:2, III:2, and III:12), three have debilitating psychiatric disease (II:7, II:9, and III:16), and two have ataxia (IV:1 and III:12), in addition to epilepsy. The eleventh affected individual (III:12) in this family had frequent seizures from the age of nine months until her death at 27 years of age. She also had severe mental retardation and her death was ruled to be caused by multiple seizures, though it is not known whether or not her symptoms were caused by the *SCN1A* mutation because her DNA was not available. Despite the relatively severe phenotype seen in most affected family members, two mutation carriers have a history of febrile seizures only (II:2 and IV:5) and one female obligate carrier (III:14) is non-penetrant for epilepsy at 35 years of age, but has a history of depression. This mutation is highly penetrant with 91% of known mutation carriers expressing an epileptic phenotype and 100% of mutation carriers displaying some neuropsychiatric spectrum disorder. The presence of an unaffected mutation carrier in this family suggests that environmental factors and/or other genetic modifiers may also play a role in the expression of the GEFS+ phenotype.

The variable expressivity in this family is compatible with the broad phenotypic spectrum previously observed in other GEFS+ families (Scheffer and Berkovic 1997; Singh, Andermann et al. 2001; Bonanni, Malcarne et al. 2004; Grant and Vazquez 2005). GEFS+ has been previously associated with wide intrafamilial variability of phenotype which can include progressive myoclonic epilepsy and developmental delay, similar to SMEI (Dravet syndrome) (Dravet, Bureau et al. 2005). Interestingly, mutations in the loop region of the *SCN1A* gene are usually associated with an SMEI phenotype, whereas in this family, though the phenotype is variable, no individual fulfills the diagnostic criteria for SMEI. The proband (IV:1) had multiple seizure types including myoclonus, in addition to developmental delay, but has not regressed (a hallmark characteristic of SMEI) and now has only occasional seizures on a single antiepileptic medication.

4.5.1 Genetic Etiology of GEFS+ and Family A

Mutations causing GEFS+ have been found in *SCN1A*, *SCN1B*, and *GABRG2* (Wallace, Wang et al. 1998; Escayg, MacDonald et al. 2000; Baulac, Huberfeld et al. 2001; Wallace, Marini et al. 2001). Missense mutations in *SCN1A* are the commonest identified molecular abnormalities and are found in about 10% of GEFS+ families (Wallace, Scheffer et al. 2001; Marini, Mei et al. 2007). Approximately 20% of cases of GEFS+ have been associated with a genetic cause (Scheffer, Harkin et al. 2005). The majority have mutations in *SCN1A*, which tend to be clustered in segments S5-S6 of the *SCN1A* protein that form the functionally critical pore region of the sodium channel. Mutations in *SCN1A* in the loop between segments S5 and S6 usually result in an SMEI phenotype (Kanai, Hirose et al. 2004). To date, there have been three other reports of mutations in *SCN1A* causing GEFS+ located in the loop between S5 and S6, all

of which are missense. The first was identified in 2001 in GEFS+ patients with febrile seizures associated with afebrile partial seizures (domain 3, S5-S6; c.4283C>T, p.Val1428Ala) (Sugawara, Mazaki-Miyazaki et al. 2001). The second occurred in a South American family in whom an amino acid substitution was located in the pore-forming region in domain four of the *SCN1A* protein (domain 4, S5-S6; c.5226C>A or G, p.Asp1742Glu). The phenotype was highly variable, with some individuals having only febrile seizures, and others having numerous seizures and mental retardation (Pineda-Trujillo, Carrizosa et al. 2005). This is consistent with the hypothesis that mutations in the critical pore-forming loop regions of the *SCN1A* protein can result in a more severe GEFS+ phenotype. However, ascertainment bias is a well-known confounder to studies of patients with more severe phenotypes. The third was identified in a single patient for whom no family history was available. At six years of age the patient has well-controlled seizures and normal cognitive and neurological development (domain 1, S5-S6; c.1130G>A, p.Arg377Gln) (Zucca, Redaelli et al. 2008).

The pores of the transmembrane protein coded by *SCN1A* control the passage of sodium ions into and out of the cell, and are thereby responsible for maintaining electrochemical balance. Mutations resulting in modifications in the pore-forming loop region may result in a significant alteration of electrochemical balance and membrane excitability controlled by this sodium channel. Although, the exact mechanism by which this mutation interferes with protein function remains elusive, further understanding of the nature and position of GEFS+ mutations will allow functional studies to develop and test hypotheses. Direct electrophysiological studies are required in order to determine the exact impact this mutation has on the structure and function of the *SCN1A* protein. *Xenopus* (frog) oocytes are commonly used for genetic expression studies of ion transport and channel physiology, due to the high degree of evolutionary conservation as

compared to human ion channels. For example, a 2006 study in California used *Xenopus* oocytes to express a novel mutation in the SCN1A protein (Nav1.1 channel) and demonstrate that the mutation caused a decrease in channel excitability (Barela, Waddy et al. 2006). This methodology could be applied to further the results of the current study by determining the effect of the novel *SCN1A* mutation on protein expression and channel excitability.

A biological continuum between neurological diseases, such as epilepsy, and psychiatric disease suggests that genes responsible for neuronal signaling may also directly affect emotional and cognitive function (Meisler and Kearney 2005). The phenotype in this family was extended to include psychiatric and neurological deficits, based largely on observations and follow-ups from home visits. It is therefore, worth emphasizing the importance of visiting families and using open-question format interviews to facilitate the collection of phenotypic data beyond the scope of structured interviews and standardized questionnaires. Increasing reports of the high degree of variability in GEFS+ families implies the presence of modifying influences including other genes and environmental factors (Ottman, Lee et al. 1996). It also presents challenges in providing affected families with effective genetic counseling and accurate information about long-term prognosis. Further studies with analysis of comprehensive medical data from GEFS+ families will help delineate the extent of neuropsychiatric features resulting from mutations in *SCN1A*.

There is remarkable clinical heterogeneity observed between affected family members which is compatible with the spectrum of GEFS+ phenotypes as described by Scheffer and Berkovic (Scheffer and Berkovic 1997). The most likely explanation for the phenotypic variability in GEFS+ families is a significant effect from genetic modifiers (none of which have yet been identified) and environmental factors. Although it is known that such factors contribute

to susceptibility, it remains unclear how they interact in their influence on disease risk and variability (Ottman, Annegers et al. 1996).

4.6 Genetic Etiology of Unsolved Families

Despite sequencing seven candidate genes known to be associated with childhood IE syndromes matching those recruited for this study, the genetic cause remains elusive in all but one family. The genetically solved family represents 12.5% (1/8) of the families with AD-compatible IE, enrolled in this study. The proportion of childhood IE cases for which the genetic cause can be explained by epilepsy-associated genes is unknown. There have been no previous population-based studies to investigate this. The genetic cause in the remaining seven AD families is likely still attributable to segregating monogenic disease alleles, yet to be identified. This may be due to differences in the genetic pool of previously studied populations and the Newfoundland population or it may be that the proportion of genetically explained monogenic epilepsies remains low due to the lack of well-ascertained, AD families segregating IE-causing mutations. It should also be considered that the etiology in patients with no family history could be attributed to a recessive inheritance pattern. Since mutation carrier parents would not exhibit symptoms, and offspring would only have a 25% chance of being affected, the likelihood of a family history in recessive conditions is relatively low. While there remain no known cases of epilepsy resulting from recessive inheritance, it is important to consider that all inheritance patterns may not yet be identified. Further to this point, *de novo* mutations could be the cause of a significant proportion of the cases with no family history. In this case, parents would be unaffected, non-carriers and the offspring would be the only affected, mutation carrier in the family. These mutations could be identified through elimination analysis of next generation

sequencing data from the two parents and the affected child. Any variants in the child that are not present in either parent are considered *de novo* and could provide a short list for potential pathogenic mutations.

Linkage analysis studies and next generation sequencing of these unsolved families fell beyond the funding parameters of this study. These types of analyses are becoming more readily accessible and affordable, and would be the most prudent course of action to undertake in further studies on these families. Also, it may be of interest to undertake testing to identify copy number variants (CNVs) in the patient population. CNVs are of particular interest in neurodevelopmental disorders such as developmental delay, intellectual disability, autism, and epilepsy. While there are only a few studies of the role of CNVs in epilepsy etiology and the underlying mechanistic pathways remain largely unknown, it would be of interest to determine any CNV patterns in a population-based study such as this one. A recent study of a cohort of 102 epilepsy patients identified 10 CNVs that were believed to be clinically relevant, including two rare deletions in novel pathogenic genetic loci (Bartnik et al, 2012). Future studies on the patient sample in the current study could employ array Comparative Genome Hybridization (CGH) to detect variations in the number of copies (duplications/deletions) of genomic segments.

4.7 Limitations

4.7.1 Challenges in genetic studies

When trying to identify pathogenic mutations using results from affected and unaffected patients, we are making two large assumptions; (1) that there are no non-penetrant mutation carriers amongst the unaffected subjects and (2) that all of the affected patients in a family share

a common mutation, thus there are no phenocopies (an individual whose phenotype for a given trait is identical to another individual whose phenotype is determined by the genotype of interest). Thus, these assumptions must be made tentatively, and the researcher must skeptically analyze the data, keeping in mind that these assumptions may not be true in all cases.

Using both affected and unaffected relatives for molecular analysis makes the assumption that unaffected relatives are non-carriers of the mutation and not that they are non-penetrant. Given that this is an early-onset disease, DNA samples from older relatives were used whenever possible, in an attempt to control for age of onset. However, most AD genetic disorders, including epilepsy, demonstrate some degree of non-penetrance. Many monogenic diseases have varied levels of penetrance depending on the mutated gene, the type of mutation, the genetic background of the family, and environmental factors. This must be taken into consideration when analyzing molecular data, particularly of unaffected subjects. An alternate model of assessing pathogenicity of a variant identified through sequencing would be to take an “affected only” approach. Those individuals unaffected would not be included in the analyses. This will address the issue of non-penetrance. Thus reducing the probability of including mutation carriers that are, as yet, unaffected. Obviously, this also reduces the “power” that one has to evaluate a potential pathogenic variant.

These approaches both incur the inherent limitation of potentially including phenocopies with the affected individuals. This reiterates the importance of obtaining the most thorough clinical data possible from all study subjects. In doing so, it aids in eliminating misdiagnoses and/or missing a diagnosis. Thus, particularly in complicated phenotypes like epilepsy, the clinical workup is instrumental to the success of a genetic study.

4.7.2 Limitations of candidate gene testing

This study was limited to the current knowledge of the genetic basis of IE. As this was a pilot project for further genetic investigations, only screening of genes previously associated with the IE phenotypes in the study subjects was performed. It is likely that many more IE-associated genes will be identified in the future. Furthermore, all IE related genes were not screened, thus it is possible that some of the previously described IE genes, not (yet) associated with the phenotypes of the patients described herein, could be causing the phenotype in these subjects. It is not uncommon for different mutations, in the same gene, to cause slightly different phenotypes. Such a phenomenon has been identified in the *RET* gene and dystrophin gene (*DMD*) which cause Hirschsprung disease and Duchenne muscular dystrophy, respectively (Edery, Lyonnet et al. 1994; Fehmann and Goke 1994; Piko, Vancso et al. 2009), among others. Thus, selecting only the candidate genes that are known to cause the phenotypes in the study subjects could limit the discovery of new phenotypes being associated with known genes.

Obviously, another limitation of candidate gene analysis is that it does not lend directly to novel gene discovery. Traditionally, it has been an important step to rule out known genes before further monetary and human resource efforts are placed into identifying novel genes. Further molecular genetic analysis, such as next generation sequencing, was beyond the budgetary scope of this project and this technology was not available until more recently.

Since the primary focus of mutation identification in this study was to Sanger sequence the coding regions and intron/exon boundaries of the candidate genes, it is possible that disease-causing variants may have been missed. There are other types of genetic variations that may be causing IE that are not amenable to discovery using the protocol outlined in this study. For example, copy number variants (CNVs), methylation abnormalities, large deletions or insertions spanning more than one exon (undetectable through Sanger sequencing in heterozygous form),

chromosomal rearrangements (such as translocations), modifier effects of variants in other genes, and functional intronic or intergenic variants would all be overlooked with the current protocol. These types of genetic variations can be examined with various other investigative approaches; however, these analyses were beyond the scope of this study.

4.7.3 *X-linked versus autosomal dominant inheritance*

X-linked inheritance refers to an inheritance pattern that arises when a gene carried on the X chromosome is mutated and causes a trait that can be passed on to the next generation. X-linked inheritance patterns can be difficult to detect when analyzing pedigrees with only clinical data available. That is, determining inheritance patterns based on pedigree data only, without knowing the underlying genotypes, often relies on educated hypotheses and thus is not ideal. Unfortunately, sometimes X-linked traits can mimic autosomal traits in some families, depending on the pedigree structure.

When a phenotype is fully penetrant and is X-linked, there are particular patterns of inheritance that can be derived from the pedigree. In conditions that are expressed in a heterozygote, all daughters and none of the sons of affected males will be affected; but if any of the daughters are unaffected and any son is affected, then the inheritance is autosomal. However, the pattern of inheritance on the X-chromosome in females mimics that of autosomes because females have two X chromosomes. Although, as a general rule, rare X-linked conditions, manifesting in heterozygotes, are twice as common in females than in males, with the caveat that the expression of most conditions is milder in females than in males. This is because males (having an XY genotype) are hemizygous (only one X chromosome is present) and do not have a "normal" X chromosome to compensate for their mutated X-chromosome.

Therefore, determining the mode of inheritance in families with an incompletely penetrant gene, causing a variably expressed phenotype, can sometimes be challenging. This is particularly true in families which are small or which only have a few affected individuals. In families with only a “triad” of affected individuals, whereby one is a first degree relative of the other two and they span two generations, there are a few scenarios in which it would be impossible to determine if a phenotype was inherited on the X-chromosome or on an autosome. For instance, if part of the triad was a mother with an affected offspring, then the mode of inheritance could be ambiguous. Or if the trio contains a father with just one female offspring, and she is affected, then it would likely be difficult to determine the mode of inheritance.

Determining inheritance patterns based on pedigree data without knowing the underlying genotypes (e.g. mutation) is often difficult, especially when families are small and/or have a small number of affected individuals, and the phenotype in question is incompletely penetrant and has variable expressivity. Unfortunately, this is a common issue when studying genetic conditions that do not fall within the category of "simple Mendelian" inheritance.

Since only clinical data was available at the candidate gene selection phase of this study, and one of the purposes of the thesis was to identify the genetic basis of epilepsy in Newfoundland, the triad was used as a surrogate for autosomal dominant inheritance. This provided a simple defining criteria, based on clinical information only, that would likely encompass all of the truly autosomal dominant epilepsy families in the cohort, but might also identify some non-autosomal families too. Due to the nature of the phenotype and the general paucity of genetic data on these study families at the time of the study start, this definition of triad was employed.

4.7.4 *Genetic classification versus clinical classification*

As research advances, we gain a more in-depth understanding into the molecular biological basis of disease. In the case of genetic disorders, over the past two decades, researchers have made immense progress into understanding the etiology of many diseases, placing medicine at a critical point in history for selecting the most pertinent system of disease classification for many disorders. The level of complexity of genetic etiology varies widely across diseases. Further, the impact of diseases on society, based on its prevalence and severity, often determines the amount of research effort dedicated to solving its genetic causes. Due to the complexity of many genetic conditions and the different levels of knowledge we have regarding them, some diseases have thus far been more amenable to a genetic classification than others. The epilepsy classification system, as set forth by the ILAE, relies on clinical data to categorize patients. An individual is classified as having a certain type of seizure and/or epilepsy syndrome based solely upon clinical presentation and findings. This system does not take into account genetic etiology. The primary advantage of using this clinical classification system is that it allows physicians to classify patients at the time of clinical workup and select the most appropriate treatment regimen specific to the seizure and/or epilepsy diagnosis. If classification was based upon genetic etiology, the majority of patients would remain unclassified as the genetic basis of IE remains unknown for most epilepsy syndromes and subtypes. Furthermore, early treatment is very important for epilepsy patients, particularly in children whose brains are still developing. Seizures which occur during this developmental phase can be potentially detrimental and may result in permanent damage. Delaying classification until a genetic cause is identified, assuming it is at all, may result in hesitation by physicians to implement the most suitable course of treatment.

However, there are also several advantages to using a genetic classification system, as indicated by other disorders that have adapted such methodology. For example, in hereditary colorectal cancer in the early 1990s, most high risk families (those with a strong family history) were clinically labeled as Hereditary Non-Polyposis Colorectal Cancer (HNPCC). Now we know that this group is largely genetically heterogeneous. However, scientists have identified one significant proportion of HNPCC families with mutations in mismatch repair genes – the cause of Lynch syndrome. Thus these families have been clinically identified as HNPCC but after genetic testing are more specifically diagnosed as Lynch syndrome. This classification has repercussions on how the “family” is treated. Those without the mutation will undergo colon cancer screening indicative of the general population while those who are unaffected and mutation carriers will be put into a more thorough screening program. Not only does this genetic classification alleviate the burden of doubt in unaffected family members but it also saves money for the health care system. Without the genetic classification, all individuals at risk for cancer in the family would have to undergo high risk screening even though approximately half of them would not have the relatively highly penetrant mutation. These are very important considerations to health care professionals when determining the implications of selecting a clinical or genetic classification system.

The challenges of implementing a genetic classification system in place of the widely used clinical system are illustrated in the example of severe infantile epilepsies. SMEI has long been known to have a genetic etiology with 50% of patients having a family history. Affected relatives of SMEI patients typically have a spectrum of symptoms such as febrile seizures and mild generalized epilepsies. These phenotypes are consistent with those in the GEFS+ spectrum. GEFS+ is associated with mutations in *SCN1A*, *SCN1B*, *SCN2A* and *GABRG2*. *SCN1A*

mutations have also been identified in a large portion (35-82% depending on the population) of SMEI cases; however, the majority of these are *de novo* mutations (Scheffer 2003). Although these molecular findings reinforce the noted clinical continuum between phenotypes of diseases with similar genotypes, they also further complicate the molecular puzzle of SMEI families. As yet, it is not fully understood why children with SMEI and *de novo SCN1A* mutations have a family history of seizures or GEFS+. It has been postulated that additional familial genes must be contributing to the seizure history in family members and these genes interact with the *de novo SCN1A* mutation in the SMEI patients to produce the more severe syndrome. Until these other genetic contributors are understood, an *SCN1A* mutation provides insufficient diagnostic information for a clinician to determine the severity of the patient's prognosis. Although identification of such a mutation supports a diagnosis of SMEI, it probably does not explain the entire picture. Since the molecular basis of SMEI is not fully understood, and complex inheritance is believed to play a role, even in *de novo* mutations, the diagnostic classification must remain a clinical one (Shorvon 2011).

4.7.5 Clinical limitations in case ascertainment

Epidemiological studies in epilepsy have a number of specific problems involving case ascertainment, classification of epilepsy, and selection bias (Sander and Shorvon 1987). Case ascertainment may pose difficulties because of deficiencies in patients' reporting and in the diagnosis of seizures, and inherent methodological problems; the classification of epilepsy is often arbitrary and definitions are often variable. Undetected selection bias may markedly influence incidence and prevalence estimates (Stuckless, 2007). The current study employed several methods to circumvent these issues. These included the use of standardized

questionnaires for seizure and family history data, using two neurologists to confirm and agree upon diagnoses, using a catchment area (the Avalon Peninsula) which would yield the highest case ascertainment, blinding chart reviews, and adhering closely to the most recent ILAE classification criteria.

Furthermore, this study employed the relevant classification criteria at the time of study inception and maintained the same parameters throughout in order to reduce selection bias and improve the power, precision and accuracy of measuring the outcomes of interest. Since the most pervasive classification system in 2003 was those which were initially set forth in 1981 and 1989 by the ILAE (with revisions in 1991 and 1993), the diagnostic utility was not entirely thorough, lacking the benefits of recent technological advances and genetic discoveries. Since the current study involved a large genetic component, the current state of literature was considered in addition to the 1989 ILAE classification system.

4.7.6 *Bias and Confounders*

Volunteer bias, also referred to as “self-selection” bias, is inherent in studies such as this where patients are allowed to refuse participation without penalty to their regular health care provision. Those who volunteer for the study may differ from those who refuse participation, thus affecting the study data. Research has shown volunteers to be better educated, healthier, lead better lifestyles, and have fewer complications given similar interventions than the general population (Stuckless S, Parfrey P, 2007). Conversely, non-response bias occurs when those who do not take part in a study differ in important aspects from those who respond. This may lead to overestimation or underestimation of the incidence of seizures in the population.

The current study made attempts to control for and measure volunteer bias. Family history of disease was assessed in responders and non-responders by blind chart review. The two groups were found to not be statistically different; thus stratified analysis was not required. However, it is difficult to control for all forms of enrolment bias, as was demonstrated in calculating the incidence. In order to determine the incidence of each epilepsy syndrome and phenotype, family members were contacted and a questionnaire was administered by telephone. If those who were willing to provide data differed from those who refused, then the incidence estimates of each phenotype may be biased. For example, parents of some patients with non-severe seizures, such as absence seizures, claimed that their child did not have epilepsy and consequently refused to answer seizure questionnaires. Thus incidence data may be skewed in favor of the occurrence of tonic-clonic and generalized seizures.

Recall bias can occur in studies such as this where subjects are required to recall past events. In this case, family members who had witnessed seizures of affected individuals were asked to recount these events and a detailed questionnaire was administered to the same witnesses for each different type of seizure the patient experienced. Patients themselves were also questioned about their experiences prior to, during (if consciousness was maintained), and after their seizures. In an effort to offset this bias, seizure types were confirmed in patient's hospital charts whenever possible. However, phenotypic information obtained using retrospective chart reviews is unlikely to unveil the detailed clinical information that can be obtained by prospective evaluation. Thus, from the cross-sectional start point of this study, all subsequent seizure events were reported to a researcher and recorded according to the ILAE questionnaire.

4.7.7 Referral bias

Making an accurate estimate of population-based incidence requires comprehensive ascertainment methods. The greatest caveat to an incidence study such as this one arises when family physicians fail to refer children with seizures to a neurologist. A study in Ontario, Canada found that $\leq 20\%$ of children with absence seizures would not be referred to a neurologist (Nixon Speechley, Levin et al. 1999). On the Avalon Peninsula of Newfoundland, standard protocol dictates that all children presenting to family physicians and emergency rooms with seizures are referred to be followed-up by a neurologist in the Janeway Neurology Clinic. In the current study, we have attempted to ascertain virtually all cases of childhood epilepsy from the catchment area of the Avalon Peninsula; however, nonreferral rates have not been assessed for this area as it is difficult to confirm the actual proportion of primary care physicians in the province that follow this route of tertiary referral.

4.7.8 Ascertainment bias

The greater incidence of IE on the Avalon Peninsula may be due to a true difference between the NL population and other published populations but also could be attributed to an ascertainment bias. Children living in close proximity to the Janeway, on the Avalon Peninsula, are more likely to be referred to a neurologist than children living in other parts of the province, where some children may be treated solely by a family physician. Thus, comparable populations may also have deficits in tertiary referral care whereby children with epilepsy are unreported in neurology clinic databases.

Further, the restrictive inclusion criteria may result in an ascertainment bias toward multiple-case families and toward a more severe phenotype. Smaller families and those with

only two affected individuals (or three affected individuals in the same generation) were not classified as being compatible with autosomal dominant inheritance leading to a possible over-representation of families with multiple IE cases included in the study sample for molecular analysis.

Subjects with a family history of seizures compatible with AD inheritance were prioritized in the candidate gene selection and screening process. This methodology is commonplace in genetic testing for pilot research projects, particularly in the case of diseases such as epilepsy where the vast majority of genes harbouring mutations have been identified in such AD families. However, this methodology invariably results in a sample set subject to ascertainment bias. Further, amongst families with a positive family history, a comparison of the number of affected relatives suggests a volunteer bias for the molecular phase of the study. That is, families with a stronger history of disease were more likely to participate in the molecular study. Since the genetic results of this study were not evaluated and generalized in comparison to other populations as the epidemiological results were, there was an inherent bias introduced via this selection method which was accepted.

4.8 *Conclusions*

The results of the current study demonstrate that the incidence of IE is high in Newfoundland, and it is associated with a high rate of familial and autosomal dominant disease as compared to other population-based studies in developed regions. It is likely that substantial genetic heterogeneity exists as judged by the clinical heterogeneity of disease and the distribution of affected families in multiple geographic isolates. Further work will involve molecular genetic studies to identify other causative genes for epilepsy in this population. A

better understanding of the genetic epilepsies at both the clinical and molecular levels will improve diagnosis, prognosis and genetic counseling for affected families, and allow the development of new strategies for pharmacological control of seizures.

As genes have been identified in several autosomal dominant epilepsy families in other populations, it is likely that other families with monogenic epilepsy syndromes are present in the Newfoundland and Labrador population (in addition to the previously identified BFNC and GEFS+ families). Clinical and genetic investigation of these families will provide information pertaining to the phenotypes and genotypes of the families as well as the modes of inheritance and clinical expression of IE in the entire Newfoundland and Labrador population.

4.9 *Future directions of epilepsy research*

As research into the molecular genetic etiology of IE continues, further epilepsy-causing genes will undoubtedly be identified. In family studies of Mendelian epilepsy, disease-causing genes are likely to be more rapidly identified through NGS. Over the past few years, numerous disease-causing genes have been identified utilizing NGS technologies that entail DNA enrichment, massive parallel sequencing and new data analyses strategies (Metzker 2010). NGS is redefining how we identify disease-causing genes. In particular, whole exome sequencing (WES) has been a very effective approach for determining the genetic cause of Mendelian disease, using just a few affected individuals (Hoischen, Gilissen et al. 2010; Ng, Buckingham et al. 2010). NGS is largely replacing the traditional method of positional cloning and candidate gene analysis for gene discovery. Instead, the coding regions (and intron/exon boundaries) of all the genes in the genome are sequenced simultaneously. All the genetic variants in these coding regions are identified then the data is systematically filtered and analyzed to sort out the benign

and/or common variants from the rare and/or disease-causing mutation(s). It is believed that approximately 80% of cases of Mendelian diseases are caused by mutations that can be detected in the exome (including intron/exon boundaries). Therefore, employing WES to continue the search for the causative variants in the remaining families would have a high probability of success.

Although it is well recognized that a single epilepsy gene can cause widely varying clinical features in different individuals in the same family, the factors determining these differences are currently not well understood. The close collaboration of clinicians and molecular geneticists will continue to provide accurate information on the clinical effects of genotypes which are identified in the population, and lead to the identification of genetic markers predisposing to specific levels of disease severity and progression. The analysis of clinical data combined with molecular genetic studies will allow delineation of natural history and genotype-phenotype correlations. This will lead to an improved understanding of the molecular genetic basis of pediatric epilepsy and more accurate prognostic information for families.

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PolyPhen SNP Data Collection <http://genetics.bwh.harvard.edu/pph/data/>

SIFT (Sorting Intolerant From Tolerant) <http://sift.jcvi.org/>

Appendices

Appendix A: Consent form for study participation

Consent Form #1: Parents of Children with Epilepsy

October 2003

Faculty of Medicine, Schools of Nursing and Pharmacy of Memorial
University of Newfoundland; Health Care Corporation, St. John's; Newfoundland Cancer
Treatment and Research Foundation

Consent to Take Part in Health Research

TITLE: A Clinical and Genetic Study of Childhood Epilepsy in Newfoundland and Labrador

INVESTIGATOR(S): Dr SJ Moore, Dr DJ Buckley, Dr MF Alam, Dr M Hall, Ms SJ
Penney, Dr P Rahman, Dr TL Young, Dr PS Parfrey,

You have been asked to take part in a research study. It is up to you to decide whether to be in the study or not. Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

The researchers will:

- discuss the study with you
- answer your questions
- keep confidential any information which could identify you personally
- be available during the study to deal with problems and answer questions

If you decide not to take part or to leave the study this will not affect you or your child's usual health care.

1. Introduction/Background:

Epilepsy is a common disorder, in which people have seizures. In about half the cases of epilepsy, the cause is not known. It is thought that genes play an important part in the disease in many of these cases.

We have about 30,000 genes in most cells in our bodies. Genes are made up of genetic material called DNA. A gene is like an instruction to the body. When some genes are changed, they can increase the risk of a person developing epilepsy.

We are doing a study to try to find out what genes are causing epilepsy in families in Newfoundland and Labrador. This would help us understand more about how epilepsy is caused. It may also help in the development of better medications for people with epilepsy in the future.

2. Purpose of study:

This study consists of two phases:

In **Phase 1**, everyone will be asked questions about their medical and family history. The purpose of this phase of the study is to understand how epilepsy genes are passed through the family.

In **Phase 2**, everyone will receive a letter explaining the way in which epilepsy is inherited in their family. Only those people in families in which epilepsy seems to be caused by a single gene will be

called again. The purpose of this phase is to discover which genes are causing epilepsy in this province.

3. Description of the study procedures and tests:

If you choose to take part in this study, you will be asked to do the following:

- Answer some questions about your health and the health of your children
- Answer questions about your family history
- Give consent for the researchers to review your medical chart, and those of your children (if they are under the age of 18 years).
- Provide a blood sample for DNA, or genetic material. This will be approximately 20 ml (1¼ tablespoons, or four small tubes).
- Consent to provide a sample of blood from your child for DNA. This will be approximately 10 ml (2/3 tablespoon, or two small tubes).
- Call some of your relatives to ask them whether they would agree to be called by a researcher about this study

You will be contacted again several months after the first telephone call to give you some feedback from the first phase of the study. If it appears that epilepsy in your family is due to a single gene being passed through the family, you will be asked to call other relatives to ask them if they would be agreeable to being called about this study.

All of these procedures will help us understand how epilepsy genes are passed through the family, and how epilepsy is caused.

4. Length of time:

You will be interviewed once or twice, each interview will last approximately 1-2 hours. You may be interviewed by telephone, or, if you attend the Janeway neurology Clinic with your child, you may be interviewed while you are attending a follow-up clinic appointment.

If you agree to have a blood sample, you would be asked to go to your local hospital to have this taken. If this is not possible, a nurse will visit you in your own home to take the blood sample.

5. Possible risks and discomforts:

There is a small risk that you may discover that you are carrying a gene which can cause epilepsy, through the genetic testing which we are doing as part of this study. A genetic result such as this would be explained to you clearly. You would be provided with genetic counseling, and you would have opportunities to ask any questions about this result.

There may be a small amount of discomfort due to the blood sampling.

6. Benefits:

It is not known whether this study will benefit you.

7. Liability statement:

Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.

8. Confidentiality:

A record that you took part in this study will be a permanent part of your health record.

When you sign this consent form you give the persons involved in the conducting the trial and the persons responsible for protecting your safety permission to inspect your health records. Only these persons may look at the health and study records that identify you by name:

- your study doctor
- the research ethics board
- Health Canada, Therapeutic Products Program (TPP)
- Food and Drug Administration, (FDA) from the USA

After your participation in this study ends, we may continue to review your records to follow your progress and to check that the information we collected during the study is correct.

Access to your records will only be allowed if your study doctor or his/her representative is present. Your name will not appear in any report or article published as a result of this study. By signing this consent form, you allow the people above to inspect your records.

If you agree, the study doctor will contact your family doctor to inform him/her that you are taking part in this study.

9. Questions:

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. Those people are:

Dr D Buckley, telephone #: (709) 777 4421

Dr S Moore, telephone#: (709) 777 4012 or (709) 777 4606

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through

Office of the Human Investigation Committee (HIC) at 709-777-6974

Email: hic@mun.ca

Signature Page

Study title: A Clinical and Genetic Study of Childhood Epilepsy in Newfoundland and Labrador

Name of principal investigator:

To be filled out and signed by the participant:

Please check as appropriate:

I have read the consent [and information sheet].

Yes { } No { }

I have had the opportunity to ask questions/to discuss this study.

Yes { } No { }

I have received satisfactory answers to all of my questions.

Yes { } No { }

I have received enough information about the study.

Yes { } No { }

I have spoken to Dr Buckley or Dr Moore and he/she has answered my questions

Yes { } No { }

I understand that I am free to withdraw from the study

Yes { } No { }

- at any time
- without having to give a reason
- without affecting the future care of myself or my child

I understand that it is my choice to be in the study and that I may not benefit.

Yes { } No { }

I agree that the study doctor or investigator may read the parts of my hospital records, and those of my child (if under 18 years), which are relevant to the study

Yes { } No { }

I agree to provide a sample of DNA

Yes { } No { }

I agree for my child (if under 18 years) to provide a sample of DNA

Yes { } No { }

If yes, please see DNA Consent Form

I agree to take part in this study.

Yes { } No { }

Signature of participant

Date

Signature of witness

Date

To be signed by the investigator:

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

Krista Mahoney
Signature of investigator

May 17/06
Date

Telephone number:

777-8567

Assent of minor participant (if appropriate):

Signature of minor participant

Date

Relationship to participant named above

Age

DNA Consent Form for Myself _____

In order to interpret the results of genetic research, we need to have correct information about parents. Sometimes the research shows new information about birth parents. This could happen in the case of an adoption or a mistake in the identity of a mother or father. This information will not be given to anyone, including yourself or family members.

In order to preserve a valuable resource, your DNA samples may be stored at the end of this research project. It is possible that these samples may be useful in a future research project which may or may not be related to the current research project. **Any future research would have to be approved by a Research Ethics Board (REB).**

Please check the box below if you are agreeable to be contacted for future research projects

<input type="checkbox"/>	I agree to be contacted for future research projects
--------------------------	--

The DNA sample from this study will be stored in St John's, Newfoundland for an indefinite period of time unless you specify that it must be destroyed at the end of the present project.

You are free to have your DNA sample destroyed at any time. If you wish to have your DNA sample destroyed at any time, please contact the Research Team, and the DNA will be destroyed by the laboratory staff. Any information extracted to this point will still be used.

Signature: _____ Date: _____

MCP: _____

Witness: _____ Date: _____

DNA Consent Form for My Child _____

In order to interpret the results of genetic research, we need to have correct information about parents. Sometimes the research shows new information about birth parents. This could happen in the case of an adoption or a mistake in the identity of a mother or father. This information will not be given to anyone, including yourself or family members.

In order to preserve a valuable resource, your DNA samples may be stored at the end of this research project. It is possible that these samples may be useful in a future research project which may or may not be related to the current research project. **Any future research would have to be approved by a Research Ethics Board (REB).**

Please tick **one** of the following options:

<input type="checkbox"/>	I agree that my child's DNA sample can be used for any epilepsy research project but <u>only</u> if I (or my child, once they reach 18 years) am contacted again to give consent for the new project.
<input type="checkbox"/>	I agree that my child's DNA sample can be used for any approved research project but <u>only</u> if I (or my child, once they reach 18 years) am contacted again to give consent for the new project.
<input type="checkbox"/>	I agree that my child's DNA sample can be used for any approved research project without contacting me again, but only if my child's name* cannot be linked, in any way, to the sample.
<input type="checkbox"/>	Under no circumstances may my child's sample be used for future research. My child's sample must be destroyed at the end of this present project.

*Includes name, MCP number or any other identifying information.

The DNA sample from this study will be stored in St John's, Newfoundland for an indefinite period of time.

You are free to have your DNA sample destroyed at any time. If you wish to have your DNA sample destroyed, please contact the Research Team, and the DNA will be destroyed by the laboratory staff.

Signature: _____ Date: _____

MCP: _____

Witness: _____ Date: _____

Appendix B: Seizure History Questionnaire

Adapted from: Reutens et al, Epilepsia 1992 33(6):1065-1071.

*** This questionnaire should be asked to all those reporting seizures, AND, if possible, a person who has witnessed the seizures, such as a parent or sibling of the person reporting seizures.**

History taken from:

Name of person to whom this history pertains:

DOB:

MCP:

Current Date:

- I. How many different types of seizures do you think you have?

- II. Please describe what happens in each type of seizure.

- III. Do you have blank spells or spells in which you go into a trance-like state?
Y / N

- IV. Do you have spells in which you twitch or jerk, especially after just waking up?
Y / N

Name of person to whom this history pertains:

For each seizure type:

Seizure Type:

- 1.0 Do the seizures follow each other in a particular pattern, for example: when wakening/ going to sleep/ with flashing lights/ around the time of the menstrual period/ or

any other pattern ?

Y / N

If Yes, describe the pattern.

2.0 Do you have any warning before seizures occur, for example, a smell or taste or different feeling, or any other warning?

Y / N

If Yes, describe the warning.

If Yes to Question 2.0:

2.1 How long does the warning last?

2.2 Does the warning ever occur on its own?

Y / N

3.0 Do you “go blank” or lose awareness of your surroundings during the seizure?

Y / N

If Yes to Question 3.0:

3.1 For how long are you blank or “out of it?”

3.2 Do you go completely blank (out of it altogether) or retain some awareness of your surroundings?

3.3 Do you stop what you are doing or continue on automatically, not being aware of your surroundings?

If No to Question 3.0:

4.0 If No to 3.0: Without losing consciousness, have your legs given way?

Y / N

4.1 Without losing consciousness, have you dropped to ground?

Y / N

4.2 Without losing consciousness, have your arms dropped or sagged?
Y / N

4.3 Without losing consciousness, have you had sudden jumps which may have caused you to drop things?
Y / N

4.4 Without losing consciousness, does your head drop or sag?
Y / N

5.0 During a seizure, do you experience emotional changes or flashbacks?
Y / N

If Yes, describe them:

5.1 During a seizure, do you experience a feeling of being in a dream or in an unusually strange or familiar place?
Y / N

If Yes, describe the feeling.

If Yes to Question 5.0:

5.2 During a seizure do you experience intense fear? Y / N

During a seizure do you experience rage or anger? Y / N

During a seizure do you experience happiness, or pleasure? Y / N

If Yes to Question 5.2:

5.3 How long do the emotional changes last?

If Yes to Question 5.0:

5.4 During a seizure do you experience flashbacks or memories of past events (as though you are reliving the past)?
Y / N

5.5 During a seizure do you experience the sense that everyday surroundings or objects are unfamiliar?

Y / N

6.0 During a seizure do you see or hear things that aren't real?

Y / N

If Yes, describe what you see or hear:

If Yes to Question 6.0:

6.2 How long does this sensation last?

7.0 During a seizure do you experience any unusual smells or tastes?

Y / N

If Yes, describe the smells or tastes.

If Yes to Question 7.0:

7.2 How long does this sensation last?

8.0 During a seizure do you notice any pins and needles, electric shocks, tingling, or other changes in sensation?

Y / N

If Yes, describe the sensations.

If Yes to Question 8.0:

8.1 Where does the change in sensation start and how does it spread?

8.2 How long does the change in sensation last?

9.0 During a seizure do objects or sounds in the room appear distorted or altered? For example, do sounds seem nearer or farther away or objects seem shrunk or magnified?
Y / N

If Yes, describe the changes.

If Yes to Question 9.0,

9.1 Indicate if auditory or visual.

9.2 How long does it last?

10.0 During a seizure do you feel that everything is in slow motion or sped up?
Y / N

If Yes, describe the feeling.

11.0 During a seizure do you experience a funny feeling in your tummy?
Y / N

12.0 During a seizure do you experience mouth watering or drooling?
Y / N

13.0 During a seizure do you experience palpitations or a pounding heart?
Y / N

14.0 During the seizure do the eyes roll back or tend to look in a particular direction?
Y / N

If Yes to Question 14.0:

14.1 Which direction do they turn?

14.2 How long does it last?

15.0 During a seizure, does the head turn in a particular direction?

Y / N

If Yes to Question 15.0:

15.1 Which direction does it turn?

15.2 How long does it last?

16.0 During a seizure, are the arms held stiff in a particular position?

Y / N

If Yes, describe what happens.

If Yes to Question 16.0:

16.1 Are both arms or is just one arm affected?

16.2 Are the arms bent or straight?

16.3 How long does it last?

17.0 During a seizure are the legs held stiff in a particular position?

Y / N

If Yes, describe what happens.

If Yes to Question 17.0:

17.1 Are both legs or is just one leg affected?

17.2 Are the legs bent or straight?

17.3 How long does it last?

18.0 During the seizure, does breathing stop or does the subject go blue? Y / N

19.0 Do any jerking or twitching movements of the face, arm, or leg occur during the seizure?

Y / N

If Yes to Question 19.0:

19.1 Which parts of the body do they affect (face or lips/arms/legs)?

19.2 Does the jerking affect both sides of the body at the same time or only one side at a time?

19.3 Are the jerks infrequent and irregular, or are they repeated and regular?

19.4 Approximately how long does the jerking last? Give approximate longest and shortest times.

20.0 During a seizure, do the eyelids twitch or is there any repeated blinking?

Y / N

If Yes to Question 20.0:

20.1 How long does it last?

21.0 During a seizure, do any of the following things occur?

21.1 Smacking of the lips

Y / N

21.2 Licking of the lips

Y / N

21.3 Chewing

Y / N

21.4 Swallowing

Y / N

21.5 Laughing

Y / N

21.6 Picking at or fiddling with things

Y / N

21.7 Walking or making stepping or bicycling movements

Y / N

21.8 Speaking

Y / N

If Yes, describe what happens.

22.0 Do you ever bite your tongue during a seizure?

Y / N

23.0 Do you ever wet yourself during a seizure?

Y / N

24.0 After a seizure are you confused or drowsy?

Y / N

Describe how you feel after a seizure.

If Yes to Question 24.0:

24.1 How long does the confusion or drowsiness last?

25.0 After a seizure, do you have a headache?

Y / N

If Yes, describe it.

If Yes to Question 25.0:

25.1 Is the headache on one side of the head or does it ache all over?

26.0 Do seizures have any longer lasting effects on vision, speech, sensation, or muscle power?

Y / N

If Yes, describe the changes.

If Yes to Question 26.0:

26.1 How long do these effects last?

Appendix C

Human Investigation Committee Application

October 2003

GENERAL APPLICATION CHECKLIST Memorial University Human Investigation Committee

One copy of the completed checklist must be attached to each copy of your application.
Please ensure all items are marked either X or NA

Short title of proposal: The Clinical and Genetic Epidemiology of Pediatric Epilepsy in Newfoundland and Labrador

- [X] Latest copy of application form has been used. (*October 2003*)
- [X] All questions have been answered in the space provided on the form or in the number of lines allowed
- [X] One copy of the application is signed by applicant
- [X] Copies of the budget are attached to *each* copy of the application
- [X] Questionnaires, chart audit forms, covering letters are attached to each copy of the application. (If standard questionnaires such as the SF36 or EROTC are used, list titles where requested and ensure one copy for the primary reviewer is included in the full protocol.)
- [X] One copy of a current curriculum vitae attached (principal investigator if first time applicant to HIC)
- [X] One copy of full protocol with signature of local investigator, is attached (*if relevant*)
Secondary use of data/tissue: One copy of data/tissue request form and one copy of letter of approval from data/tissue guardian is attached.
- [X] I have read "Guidelines for Preparation of a Standard Consent Form"
- [X] *Consent document is attached (written consent using HIC template or script for verbal consent) to each copy of application*
- [X] DNA/ tissue consent included, if relevant.
- [X] Consent has been assessed at a reading level of grade 8 (must be less than grade 9).
- [X] I have verified that the above information is correct:

Name of Principal Investigator(s): Dr. SL Moore Dr. DJ Buckley

HUMAN INVESTIGATION COMMITTEE

General Application Form

October 2003

Please complete the application in **bold** or a font which can be easily distinguished from the application form. Applicants are advised to consult Application Guidelines

Forward one copy of the checklist, application, budget, consent form and any other documents (questionnaires, scripts, etc.) for screening to the Human Investigation Committee (HIC) Office, Room 1755/57, Health Sciences Centre, Phone: 777-6974. Twenty-four copies, submitted in sets, will be required by HIC when the application has been screened and allowed to proceed to review.

1. Investigators: Dr SJ Moore, Dr DJ Buckley, Dr MF Alam, Ms SJ Penney, Dr P Rahman, Dr TL Young, Dr PS Parfrey, K Mahoney

• Principal Investigators:

Last Name, First Name, Title (Dr./ Mr./ Ms./ Mrs.)

Moore, Susan Dr

Buckley, David Dr

(a) Faculty

[X] Dr Buckley

(b) Employee of HCCSJ, NCTRF

[]

(c) Undergraduate, graduate, postgraduate student

[]

(c) Other: *[please specify]*

**[X] Dr Moore, Clinical Research
MUN**

Associate,

• Mailing Address:

Internal: Pediatric Neurology

Janeway Child Health Centre - Dr Buckley

Clinical Epidemiology

Health Science Centre - Dr Moore

External:

Street Name & #

300 Prince Philip Drive

P.O. Box City / Town

St. John's, NL

Postal Code

A1B 3V6

- Telephone Number: **777 4421 Dr Buckley**
777 7616 Dr Moore
- Pager Number:
- Email Address: DrBuckley:hcc.bargerm@hccsj.nf.ca; Dr Moore: smoore@mun.ca
- Co-investigators:

Dr MF Alam, Ms SJ Penney, Dr P Rahman, Dr TL Young, Dr PS Parfrey

- Local contact (name and contact information) if principal investigator is external:
- Research Coordinator (if relevant): Ms S Penney or Krista Mahoney

Telephone Number: 777 4172

Email: kmahoney@mun.ca

2. Title of study:

A Study of the Clinical and Genetic Epidemiology of Pediatric Epilepsy in Newfoundland and Labrador .

3. Study timeline:

- Proposed start date *[at least 4 weeks from date of submission].* '04/01/2004
- Anticipated completion date: 04/01/2005
- Deadline for ethics approval: 03/01/2004

Indicate below if:

[] course project

4. Setting of study and data sources:

- Setting - Please specify the institutions and/or communities involved: **Patients will be recruited through the Janeway Child Health Centre Neurology Clinic.**

- Check relevant data sources:

(a) Patients [X]

(b) Health Providers []

(c) Clinical Records [X]

(d) Pre-existing Dataset [X] **The Janeway Neurology Clinic Epilepsy Database will be used to ascertain patients. This is run by Ms SJ Penney, Pediatric Neurology Research Nurse, Janeway Neurology Clinic, phone #: 7097774172**

(e) Residents in Community []

(f) Archived Specimens []

(g) Other: *[please specify]*

5. Objectives:

- To describe the incidence and clinical epidemiology of pediatric epilepsy over the last five years in Newfoundland and Labrador, using a defined sub-population on the Avalon Peninsula. This will be achieved using data currently stored on the Janeway Neurology Clinic database.
- To describe the genetic epidemiology of idiopathic epilepsy, and identify genes involved in idiopathic epilepsy in the province. This part of the study will involve families from any part of Newfoundland and Labrador.
- To assess whether there is a correlation between genotype and phenotype in idiopathic epilepsy, and identify genetic markers of severity and progression.
- To determine whether there are genotypes which are associated with resistance to antiepileptic medication in idiopathic epilepsy.

6. Introduction to the study:

The average incidence rate for epilepsy per 100,000 children is reported as approximately 40 to 80 (Camfield et al, 1996; Blom et al, 1978). Approximately 40% to 60% of all epilepsy is idiopathic (Weissbecker et al, 1999). Family studies have shown a risk to the sibling of an affected person with epilepsy is approximately 2.5 to 4 fold greater than the general population risk (Annegers et al, 1996; Weissbecker et al, 1999). The heritability of epilepsy has been estimated as 70% to 88% (Kjeldsen et al, 2001). Most cases of idiopathic epilepsy (IE) follow complex inheritance, involving several genes and environmental factors (Berkovic et al, 1990, 1998; Greenberg et al, 1992). Less often, epilepsy can result from a single disease gene. Genes have been identified in several autosomal dominant epilepsy families (Baulac et al, 2001; Beivert et al, 1998; Charlier et al, 1998; Cossette et al, 2002; Phillips et al, 1995; Singh et al, 1998; Steinlein et al, 1995; Sugawara et al, 2001; Wallace et al, 1998). Genetic heterogeneity has been shown for all of these epilepsy syndromes. Variable expressivity also occurs, implying that modifying genes exert an important effect on the phenotype, though these have not yet been identified (Scheffer and Berkovic, 1997; Wallace et al, 1998). Attempts to identify disease genes in the majority of cases of IE, which follow complex rather than monogenic inheritance, have

been less successful. Several loci have been identified, but these findings have not been replicated. Juvenile Myoclonic Epilepsy (JME), one of the commoner IE subtypes, has been associated with a locus on 6p (Durner et al, 1991; Greenberg et al, 1988; Liu et al, 1995; Weissbecker et al, 1991) but this was not confirmed by other groups (Elmslie et al, 1996; Whitehouse et al, 1993). The apparently contradictory evidence from association studies of epilepsy implies that ethnic factors and genetic heterogeneity are major confounding variables in these studies.

Rationale for the study:

Epilepsy is a common, chronic neurologic condition which creates a significant burden for the affected child, their family and society. Idiopathic epilepsy is known to have a genetic basis, though little is known about which genes cause the disease in most cases. One of the epilepsy genes which causes Benign Neonatal Febrile Convulsions (BNFC) was identified through genetic linkage studies on a large Newfoundland kindred (Singh et al, 1998). It is likely that other families, with mendelian inherited epilepsy syndromes are present in the population of Newfoundland and Labrador. Clinical and genetic studies of these families will provide valuable information not just for the families themselves, but also for the wider pediatric epilepsy community. The analysis of thorough clinical data combined with molecular genetic studies will allow delineation of natural history and genotype-phenotype correlations. This will lead to an improved understanding of the molecular genetic basis of pediatric epilepsy, and more accurate prognostic information for families.

Molecular genetic studies of those families with complex inheritance of pediatric epilepsy are more likely to be successful in the population of this province than in other more admixed populations. The decreased genetic heterogeneity combined with extended linkage disequilibrium present in this relatively isolated population increases the power of association studies to detect disease loci. These findings would help identify the genes which contribute to the majority of cases of pediatric epilepsy, inherited in a complex manner, in the province, and improve the understanding of the pathogenesis of this common disorder.

7. Blood or other tissue sampling which is part of the study: Not Applicable []

- List samples to be taken from participants, the frequency of sampling and the amount of sample.

One blood sample will be taken for DNA extraction: 20ml from adults, 10ml from children, and 5ml from infants.

- Will any samples be kept after the completion of this study? Yes [X] No []
- Can participants withdraw their blood, tissue or other sample? Yes [X] No []
- Will any samples now archived by a health care institution be used in the study?

Yes [] No [X]

8. Research interventions and/or modes of data collection:

- List any interventions which would not be part of a participant's daily life.

All participants will be asked to:

1. Provide a family history
2. Provide a medical history

In addition, affected children, their parents and some other participants (in families showing mendelian inheritance of epilepsy) will be asked to:

3. Provide a blood sample for DNA extraction.

Also, controls recruited from the Health Sciences Centre Blood Collection Laboratory will be asked a few questions about their medical history, and asked to provide a sample of blood for DNA extraction.

- List questionnaires, information sheets, covering letters, telephone or face to face interview scripts/outlines or chart audit forms to be used. Include copies of each with each copy of the application; if standard questionnaires are being used - SF36, EROTC, etc. [*see list on HIC website*] include one copy only.

1. Telephone or face to face interview script for initial contact with all parents of affected children: Appendix 1a
2. Cover letter (for parents of affected children): Appendix 1b
3. Cover letter (for relatives other than parents of affected children, contacted during Phase 1): Appendix 1c
4. Medical Questionnaire (used for all participants): Appendix 2
5. Chart audit form (used only for those participants in families showing mendelian inheritance of epilepsy): Appendix 3
6. Standard seizure questionnaire (used for all participants reporting a history of seizure): Appendix 4
7. Laboratory Requisition Form (used for all parents of affected children, and other relatives from whom DNA is requested): Appendix 6
8. Letter to families showing complex inheritance of epilepsy: Appendix 8

9. Letter to families showing Mendelian inheritance of epilepsy: Appendix 9

10. Interview script for initial contact with potential controls in Blood Collection Laboratory of the Health Sciences Centre: Appendix 10

11. Medical Questionnaire for controls, recruited from the Blood Collection Laboratory at the Health Sciences Centre: Appendix 11

9. Description of study:

For Protocol, see Appendix 5.

The study to identify epilepsy genes will take place in two phases.

First Phase

The first phase is concerned with data collection and pedigree analysis to identify the inheritance pattern in the family.

A medical history will be taken from all participating parents of children with idiopathic epilepsy. A medical history will also be taken for siblings of the affected child (from the parents), and from the grandparents (for themselves, and all their offspring). If available, great-grandparents of the affected child will be asked for a medical history of themselves and their offspring.

Blood samples will be taken from the affected child and their parents for DNA extraction. Genetic counseling will be provided by the Research Nurse employed for this project, who will be trained in this area.

During this time, controls will be recruited from the Blood Collection Laboratory of the Health Sciences Centre. They will be asked a short medical questionnaire, and asked for a sample of blood for DNA extraction, after genetic counseling.

Second Phase

The second phase involves molecular genetic analysis of DNA from families. The method of genetic analysis for each family will be determined by the results of data collected during Phase 1.

Family history data collected during Phase 1 will be analysed, and families will be categorised as having complex or Mendelian (for example autosomal dominant or recessive) inheritance of epilepsy. Letters will be sent to all participants to inform them of the pattern of inheritance of epilepsy in their family.

- Parents of affected children from families with pedigrees suggestive of Mendelian inheritance will be contacted again and asked to participate in genetic linkage studies to try to identify the disease gene causing epilepsy in the family. At this time,

medical histories will also be taken from the affected child's parents' siblings, and spouses married into the family. Medical histories will be confirmed by review of medical charts.

Blood samples for DNA extraction will be taken from as many relatives as possible. When available, results will be given to relatives, with genetic counseling.

- Participants in families showing complex inheritance of epilepsy will not be called again. Genetic association studies will be performed on DNA from these participants. These will involve comparing patterns of DNA around known epilepsy genes in affected children and controls, to determine whether particular epilepsy genes are associated with idiopathic epilepsy in this province.

Note: Phase 2 may be started before Phase 1 is completed, if families showing Mendelian inheritance of epilepsy are identified during the course of Phase 1.

10. Sample size: [if measuring statistical differences/equivalencies]

1. Sample size calculation is not relevant for the part of this study which involves genetic linkage studies in the families showing Mendelian inheritance of epilepsy. A family with multiple affected individuals and several unaffected relatives from whom DNA is available, would be eligible for this analysis.

2. For the group of families showing complex inheritance of epilepsy who will be analysed using the case control method and single nucleotide polymorphism (SNP) technology, the sample size is calculated as follows:

Null Hypothesis: For a particular gene with two alleles, either allele will be transmitted randomly to affected offspring. On average, an allele will be transmitted in 50% of cases, so expected proportion $p=0.5$

Alternative Hypothesis: An allele (which predisposes to epilepsy) will be transmitted on average in 60% of cases to affected offspring.

In complex inheritance, several different genes each contribute a small predisposition to the disorder, so a difference of 10% in the rate of transmission of a predisposing allele from parent to affected child would be reasonable to assume.

Thus, the expected proportion of a particular allele in the affected children, $p=0.6$
The 95% confidence interval (CI) should not overlap 0.5 (expected proportion for the null hypothesis, which would be the expected proportion in the control group), so use a half width of the CI for a two-tailed test of .07.

Use the following formula to calculate sample size, N:

$$N = p(1-p) \times (1.96 / \text{desired half width of CI})^2$$

p = expected proportion, for alternative hypothesis, = 0.6

$1-p$ = 0.4

desired half-width of CI = 0.07

=> = **188** = number of cases of children affected with idiopathic epilepsy from whom DNA is available

We will attempt to get parents of probands whenever possible to facilitate linkage disequilibrium studies.

11. Participants:

- Describe the participants to be contacted or whose record information will be used.

(a) Adults [X]

(b) Children under 19 [X]

(c) Persons incompetent to give consent []

(d) Protected or vulnerable populations []

This is a study of pediatric epilepsy, so the affected population are children.

- Number of participants at this site: approximately 400

• Will pregnant women be excluded? Yes [] No [X]

• Is this a part of a national/international study? Yes [] No [X]

- Will contact be made with potential participants? Yes [X] No [] records only

(a) Attending physician [X]

(b) Investigator [X] *[See guidelines]*

(c) Other: Research Nurse or coordinator [X]

1. For parents of children affected with epilepsy (and the children themselves if they are old enough to understand) first contact will be made by Ms S Penney, Dr Buckley or Dr Alam. If the individual agrees to be contacted by a researcher, the Research Nurse will then make contact by telephone to explain the study, and information sheets and consent forms will be sent by mail.

2. In the case of relatives of the affected child other than the parents, these people will not be contacted until the parents have asked them if they are happy to be contacted by a researcher about the study. Once it is confirmed that they are agreeable to be contacted, the Research Nurse will contact them by telephone and send information sheets and consent

forms by mail.

In both scenarios detailed above, people to whom the information and consent forms have been mailed will be called again by telephone three weeks after mailing if the signed consent form has not been received, and any questions will be answered. If the individual does not wish to participate at this time, they will not be called again.

Once the signed consent form is received, the individual will be called by telephone and interviewed, using questionnaires shown in Appendix 2, and Appendix 4 if appropriate.

During the study, approximately 200 controls will be recruited from adult patients presenting to the Blood Collection Department at the Health Sciences Center. These patients will be asked a short questionnaire to check they do not have a personal or family history of seizures (Appendix 11), and asked to provide a sample of blood for DNA extraction. These samples will be used anonymously as controls in association studies (see Protocol in Appendix 5).

12. Consent process:

- Who will obtain the consent? Research Nurse, who will be employed for this project, Dr S Moore, or K Mahoney

See Section 11.

13. Risks, discomforts and inconveniences:

- There will be inconvenience and minor discomfort associated with having blood samples taken for DNA extraction. However, inconvenience will be minimized by using local hospital laboratories for this purpose, and if this is too difficult for the individual, a phlebotomist will visit their home to take the blood sample.
- There is a risk that a person will be identified as being a carrier of an epilepsy gene, if they are in a family showing Mendelian inheritance of epilepsy, and the disease gene is identified. These people will be referred to the Newfoundland and Labrador Medical Genetics Program for further genetic counseling.

14. Benefits:

- Are there any immediate benefits for participants, including controls?
No

15. Privacy and confidentiality:

- What steps will be taken to protect privacy and confidentiality of information?

- (a) Oath of confidentiality [X]
- (b) Locked storage [X]

- (c) Limited access ☒ [X]
- (d) Password-protected computer files ☒ [X]
- (e) Denominalized files provided by data holder to investigator ☐ []
- (f) Coded study number ☒ [X]
- (g) Locked room ☒ [X]
- (h) Anonymous responses to investigator ☐ []

Study codes will be used on all samples sent to laboratories involved in the molecular genetic analysis, no identifying information will be provided.

- List below the names of all personnel who can access the identities of study participants:
Ms S Penney, Dr D Buckley, Dr MF Alam, Dr SJ Moore, Krista Mahoney, Research Nurse.

16. Debriefing:

When the pedigrees have been analysed, a letter will be sent to all families explaining the inheritance pattern of epilepsy in their family (Appendix 8 and 9). For those families showing mendelian inheritance of epilepsy, molecular results will be given to family members, with genetic counseling.

The results of the study will be submitted for peer-reviewed publication in an appropriate medical journal, and will be presented at scientific meetings.

17. Payments:

- Do you intend to reimburse participants for expenses incurred?
Yes ☒ [X] No ☐ [] Amount [\$]

18. Budget: Not Applicable ☐ []

The budget in this application refers only to Phase 1, since the scope of work required for Phase 2 will not be known until data collection has been completed. A further submission will be made with a budget for Phase 2 in due course.

Research Nurse

The research nurse will:

- take pedigree and medical information from families who have agreed to participate
- put pedigree information onto computer
- arrange blood sampling for DNA extraction from those who have consented

37.5 hours per/week for 1 year (\$30.14 per hour) \$58,783.24 + 20% benefits = \$70,539.89

Expenses for travel, meals, etc.

For participants who travel to the Janeway Child Health Centre especially for participation in this research. This does not apply to those participants whose appointments for the research study are arranged to co-incide with Neurology Clinic

follow-up appointments.

100 people x \$15/person \$1500

Expenses for phlebotomy services

For participants who live in remote areas and require a visit by a local phlebotomist

100 people x \$30/visit \$3,000

Overhead Expenses:

Postage, communications (telephone, fax), office supplies \$5400

TOTAL COST \$80,439.89

• Source of funding: An application has been made to the Janeway Foundation. Further grant applications are being made to other institutions, including CARR, Health Care Foundation, Epilepsy Newfoundland and Labrador.

• Will the budget be administered through the University Finance Office? Yes [X] No []

If no, please specify the person or agency responsible:

19. Potential conflict of interest:

• Is any investigator a shareholder in any company/agency funding this study? Yes [] No [X]

• Will any investigator receive direct financial or other benefit? Yes [] No [X]

If yes, please describe:

• Will any investigator receive indirect financial or other benefit? Yes [] No [X]

{share of profits, future royalties, patent rights, et all

If yes, please describe:

20. Ownership, storage and destruction of data:

• The investigator must be free to publish within 6 months after submitting the manuscript to the sponsor for review. Publication of the full study must be assumed no longer than 1 year after the completion of the study. In agreement with the Office of Research, HIC will assume these terms will be negotiated in any research contract.

• Do you intend to destroy the data collected at the end of the study? Yes [] No [X]

If no:

(a) Please give the anticipated date of destruction: Results of molecular genetic testing will not be destroyed. If family members ask for further testing in the future, these results may be helpful. DNA will not be destroyed unless the individual requests this, since it may be needed for further tests in the future.

(b) In what form will the data be retained, e.g., frozen samples, computer tapes, paper? Molecular genetic results will be stored on a password protected computer, and in charts which will be stored in locked cabinets. DNA samples will be stored in the Medical Genetics Laboratory, Health Sciences Centre, St John's.

(c) Where will the data be stored?

Data from the study will be stored in the Patient Research Centre.

In those families showing Mendelian inheritance of epilepsy, molecular genetic results will be:

- sent to the family member.
- kept in a Clinical Genetics chart, stored in the Genetic Clinic
- in the case of the affected child, kept in the child's Janeway Neurology Clinic chart

(d) Who will be the data guardian?

Name: Dr S Moore, and, for those individuals who are given molecular genetic results, health care professionals in the Janeway Neurology Clinic and Genetics Clinic.

Contact information:

- Will any form of identifier - name, postal code, study code, be retained? Yes [X] No []
- If yes, please describe the identifiers to be retained and give the rationale for their retention. Identifying details including MCP, date of birth and address, will be retained. This is necessary in order to identify the individual to whom a molecular genetic result pertains. However, no identifying information will be sent to the laboratories in which the genetic analyses are being undertaken.

21. Concurrent submissions or approvals:

Has this proposal been submitted to another REB? No. If already approved, please include one copy of approval.

Reminders:

- *The use of personnel and/or resources of the Health Care Corporation of St. John's requires the approval of the Research Proposal Approval Committee (St. John's)*

subsequent to HIC approval. Such approvals may also be required by institutions outside the HCCSJ and/or by regional health boards.

- Forward one copy of the checklist, application, budget, consent form and any other documents (questionnaires, scripts, etc.) for screening to the Human Investigation Committee (HIC) Office, Room 1755, Health Sciences Centre, Phone: 777-6974/7719. Twenty-four copies, submitted in sets, will be required by HIC when the application has been screened and allowed to proceed to review.

Signature of principal investigator:

Date:

December 1, 2003

Signature of undergraduate/graduate/postgraduate student (*if applicable*):

Krista Mahoney

A handwritten signature in cursive script that reads "K. Mahoney". The signature is written in a light grey or blue ink.

Appendix D

Primer Sequences

A) Microsatellite Marker and SNP Primer Sequences

Gene/ primer name	Direction	5' Modification	Primer
SCN1A SNPs			
SCN1A-Ex9-F	Forward		acagggtgctatgaccaaca
SCN1A-Ex9-R	Reverse		tcctcatacaaccacctgctc
SCN1A-Ex15-F	Forward		gagacgggttagggcagatca
SCN1A-Ex15-R	Reverse		tatacatgtgccatgctggt
SCN1A-Ex26-F	Forward		ccttggagcctttagaagaaaca
SCN1A-Ex26-R	Reverse		GGTTGTAATTTGGAATAGGCAGA
SCN1A Microsatellites			
D2S2330-F	Forward	6-FAM	AGTCATCTTATAGAAAATAGCAAGC
D2S2330-R	Reverse		AGTTTACAACTTAATTNTTGGGTG
D2S382-F	Forward	6-FAM	ATGCTAAATCTCAAAGCCAGTCT
D2S382-R	Reverse		CTAATNCTCAATTCAGTGCTCCT
D2S156-F	Forward	6-FAM	CCCTCTGTTTGCCTGAAATA
D2S156-R	Reverse		CCAGCCTTGATTGTCACAC
D2S399-F	Forward	6-FAM	CAAACATCCTACCTGCACAG
D2S399-R	Reverse		CTCAATGGGAAGTTATTGAATG

B) Primer Sequences by Exon

Gene/ primer name	Direction	Primer
<i>SCN1A</i>		
NM_006920_Ex1_F	Forward	tttaaatcctctagctcatgtttca
NM_006920_Ex1_R	Reverse	TTGGCATGTGTTGGTGCTAC
NM_006920_Ex2_F	Forward	cagtgcagttgttttcattgg
NM_006920_Ex2_R	Reverse	tgaggctaataatgacaaagatgc
NM_006920_Ex3_F	Forward	tttgggctttcaatgttagc
NM_006920_Ex3_R	Reverse	tgctgcgtaattttgtctagga
NM_006920_Ex4_F	Forward	ctgatggagtataaagaaattgg
NM_006920_Ex4_R	Reverse	ttctacaggtaaagcaaacctattc
NM_006920_Ex5_F	Forward	aagaagtgattagagtaaaggataggc
NM_006920_Ex5_R	Reverse	caagcagagaaggatgctga
NM_006920_Ex6_F	Forward	ctccactagcgttgcaaca
NM_006920_Ex6_R	Reverse	ggatatccagccctcaagt
NM_006920_Ex7_F	Forward	aaatacttgctcctttgaatgaa
NM_006920_Ex7_R	Reverse	gttggctgttatcttcagtttctat
NM_006920_Ex8_F	Forward	ccaaatctttctgactaggcaat
NM_006920_Ex8_R	Reverse	caagtctcgtttcaagttctgc
NM_006920_Ex10_F	Forward	aagccatgcaaatacttcagc
NM_006920_Ex10_R	Reverse	cacaacagtgggtgattcagttg
NM_006920_Ex11_F	Forward	tgaatgctgaaatctccttctaca
NM_006920_Ex11_R	Reverse	gctgggtgcagcaatagttag
NM_006920_Ex12_F	Forward	atctgccatcaccaattgaa
NM_006920_Ex12_R	Reverse	tgactattccaactcacaa
NM_006920_Ex13_F	Forward	aaggttgctctgtacaacaaatgtg
NM_006920_Ex13_R	Reverse	caggaagcatgaaggatggt
NM_006920_Ex14_F	Forward	gaatcattgtgggaaaatagca
NM_006920_Ex14_R	Reverse	gctaattggtgtgtggcaaa
NM_006920_Ex15_F	Forward	tgaccatttctaggtaaagctcaat
NM_006920_Ex15_R	Reverse	tttgacaaaacagtcaccattaaa
NM_006920_Ex16a_F	Forward	gaatgggagaacttgagagca
NM_006920_Ex16a_R	Reverse	TTTCCAATTTCTGCTGTATGA
NM_006920_Ex16b_F	Forward	TGGATAGGATGCACAAAGGA
NM_006920_Ex16b_R	Reverse	tcaaaacatcataagctgagga
NM_006920_Ex17_F	Forward	aaaagggttagcacagacaatga
NM_006920_Ex17_R	Reverse	ttgggcagatataatcaaagca

NM_006920_Ex18_F	Forward	gctagcaagacagagatgagca
NM_006920_Ex18_R	Reverse	gggataattaaaaatgcattgga
NM_006920_Ex19_F	Forward	ctgccctcctattccaatga
NM_006920_Ex19_R	Reverse	tctgtcaacataacatagtggctga
NM_006920_Ex20_F	Forward	aaaggtttactgaaaatgtctgaaca
NM_006920_Ex20_R	Reverse	ttgcaagaatttgccattcc
NM_006920_Ex21_F	Forward	tggaaagaccagagattactaggg
NM_006920_Ex21_R	Reverse	cacctatctgggctcataaa
NM_006920_Ex22_F	Forward	tgtctttcaaaaatattcccttttg
NM_006920_Ex22_R	Reverse	ttggtcgtttatgctttattcg
NM_006920_Ex23_F	Forward	gcacattactgttcaaatggaag
NM_006920_Ex23_R	Reverse	ttcttagctttctatagccggaaa
NM_006920_Ex24_F	Forward	ttgggggtgtttgtcttcat
NM_006920_Ex24_R	Reverse	tggattgtaatgggggtgctt
NM_006920_Ex25_F	Forward	caaaaatcagggccaatgac
NM_006920_Ex25_R	Reverse	tgattgctgggatgatcttg
NM_006920_Ex26nc1_F	Forward	CCTATGACCGGGTGACAAAG
NM_006920_Ex26nc1_R	Reverse	CCCAGGTGGCATACTGTTA
NM_006920_Ex26nc2_F	Forward	GGGTGCAAACCTGTGATTTT
NM_006920_Ex26nc2_R	Reverse	TGACTCCAAACATTTGAATGAA
NM_006920_Ex26nc3_F	Forward	GGGTGATAAGTGGAGGTGCT
NM_006920_Ex26nc3_R	Reverse	TGAACAATTTCTTGACTTTACCA
NM_006920_Ex26nc4_F	Forward	TGGATGCTATCAATGCATGTTT
NM_006920_Ex26nc4_R	Reverse	TTTGGATATGAATCGTGAACCT
NM_006920_Ex26nc5_F	Forward	CATGAAAACAAGACTGGGTAGTTG
NM_006920_Ex26nc5_R	Reverse	cccttctccccaatttgta
KCNQ2		
NM_172107_Ex6variant_F	Forward	gcccttcgtgtgactagagc
NM_172107_Ex6variant_R	Reverse	gggtccacctagggaactgt
KCNQ2_Ex1_F	Forward	tccctttccctctctctcc
KCNQ2_Ex1_R	Reverse	tacaatcgctctccggctctc
KCNQ2_Ex2_F	Forward	ctatgggggtgccctctg
KCNQ2_Ex2_R	Reverse	gcgtcagaggccctgtagta
KCNQ2_Ex3_F	Forward	ggtcagcttgcagtttcctt
KCNQ2_Ex3_R	Reverse	aacttcagaaggagccagtc
KCNQ2_Ex4_F	Forward	tctgcacatcctcctggaac
KCNQ2_Ex4_R	Reverse	cactgactccatccctccac
KCNQ2_Ex5_F	Forward	tcatgggtgttgatgtgatgg

KCNQ2_Ex5_R	Reverse	gccagtgagagcctggtc
KCNQ2_Ex6_F	Forward	gcattgtgccatcccaag
KCNQ2_Ex6_R	Reverse	gggtccacctaggaactgt
KCNQ2_Ex7_F	Forward	gggtgtgtgagtctctggagt
KCNQ2_Ex7_R	Reverse	cttcacatcctcgctccttc
KCNQ2_Ex8_F	Forward	agtgcctggcctacagcat
KCNQ2_Ex8_R	Reverse	tgaacaacaaaaagtggggttt
KCNQ2_Ex9_F	Forward	agaaccagcccttgtgctaa
KCNQ2_Ex9_R	Reverse	ccggtcacagttccagaca
KCNQ2_Ex10_F	Forward	gtgcaagcagaggagggt
KCNQ2_Ex10_R	Reverse	actgtctctggcgtgtctct
KCNQ2_Ex11_F	Forward	tgacctatgggactgtgg
KCNQ2_Ex11_R	Reverse	acacaaggccctcacatctc
KCNQ2_Ex12_F	Forward	gcagagtgacttctctccctgt
KCNQ2_Ex12_R	Reverse	gttccactccaagagcaag
KCNQ2_Ex13_F	Forward	gctctgtctagccggtgtg
KCNQ2_Ex13_R	Reverse	gctctgtccagcaccatga
KCNQ2_Ex14_F	Forward	ccgaggtctcagagggtgcta
KCNQ2_Ex14_R	Reverse	acacaacagaagctgacagagg
KCNQ2_Ex15_F	Forward	ctagaccaggggcactgt
KCNQ2_Ex15_R	Reverse	agagcagctgtcagtgggtga
KCNQ2_Ex16_F	Forward	tgggtctctctgtttctctg
KCNQ2_Ex16_R	Reverse	GGGGTACAGAGGTTCGGAGTC
GABRA1		
GABRA1_Ex1_F	Forward	cctaccatccgtcaccattc
GABRA1_Ex1_R	Reverse	ggtgagagacatacggactgc
GABRA1_Ex2_F	Forward	tcaccgcctatttcacatcc
GABRA1_Ex2_R	Reverse	cccccgactataacattcctg
GABRA1_Ex3_F	Forward	tcagtcagccctgggtgt
GABRA1_Ex3_R	Reverse	caattttcttccccgataaa
GABRA1_Ex4_F	Forward	gagaaaggattttatttgaaagagg
GABRA1_Ex4_R	Reverse	tgtcaccattttattatttgacca
GABRA1_Ex5_F	Forward	gggtgcagtatgtggtggtga
GABRA1_Ex5_R	Reverse	gccaagacttttcaggttgc
GABRA1_Ex6_F	Forward	gcaaaaattatgcactgtctgc
GABRA1_Ex6_R	Reverse	attccaccatggctcatca
GABRA1_Ex7_F	Forward	gctacagttaataacattccactgt

GABRA1_Ex7_R	Reverse	acgcgtttttctcacagatatag
GABRA1_Ex8_F	Forward	tctttcctagcctgccctct
GABRA1_Ex8_R	Reverse	gctcaactgatgcccaattt
GABRA1_Ex9_F	Forward	ttcccagacctttggactcat
GABRA1_Ex9_R	Reverse	tgagagtggcaattcctga
GABRA1_Ex10_F	Forward	tcaagtaggctgtcccatca
GABRA1_Ex10_R	Reverse	accacaggccaaaacatctc
GABRA1_Ex11_F	Forward	aaaataagggccaccttget
GABRA1_Ex11_R	Reverse	ATCCTGTCTCCAAGCTGCAT
SCN1B		
SCN1B_Ex1_F	Forward	ggtgcacctagcggatgt
SCN1B_Ex1_R	Reverse	caccctcacctccactgc
SCN1B_Ex2_F	Forward	atccagtctgtctgtctggt
SCN1B_Ex2_R	Reverse	caggtcagcaatcacagcat
SCN1B_Ex3_F	Forward	ggggacagaatcagggtca
SCN1B_Ex3_R	Reverse	ccactgcctgccatctgt
SCN1B_Ex4_F	Forward	aagctcacacagcaagctca
SCN1B_Ex4_R	Reverse	ggtctgacgaccctatcct
SCN1B_Ex5_F	Forward	tgtgtacctgggggtggagac
SCN1B_Ex5_R	Reverse	gcaagagagggggaattagg
SCN1B_Ex6_F	Forward	cagaggggaaggggattg
SCN1B_Ex6_R	Reverse	ggcactaagtggcttcacct
SCN2A		
SCN2A_Ex1_F	Forward	atgccattttgtagtcctgt
SCN2A_Ex1_R	Reverse	agagggaccactttcatttt
SCN2A_Ex2_F	Forward	TCCCTCCCTGTTTCTGTAGC
SCN2A_Ex2_R	Reverse	GCCCATCCAGCATCTTAGTG
SCN2A_Ex3_F	Forward	GCTTTATGTGAATTATAAATGCGG
SCN2A_Ex3_R	Reverse	TGTGGCAAGCAATTCGTAAG
SCN2A_Ex4_F	Forward	AACTTCATGGTGGTGAAGGC
SCN2A_Ex4_R	Reverse	AAATTGAGGAGGTCTCAAGGTG
SCN2A_Ex5_F	Forward	GGGACCAACCTGGAAGTGTC
SCN2A_Ex5_R	Reverse	TCAAGGCAAGCCACCAAG
SCN2A_Ex6_F	Forward	CCAAGGAAGACGTAGATTTC
SCN2A_Ex6_R	Reverse	AAGCAAAGAATCAAATCCATCC
SCN2A_Ex7_F	Forward	TGCTTTCATTCAATTTACCAG

SCN2A_Ex7_R	Reverse	CAGTCTTCTTAACACATTTTCAGTGG
SCN2A_Ex8_F	Forward	AGCTTTGCCACCTAAACAGG
SCN2A_Ex8_R	Reverse	GAGTGGGACTGGATGAGTGG
SCN2A_Ex9_F	Forward	GCCTGTATAAAACAGACATTGGC
SCN2A_Ex9_R	Reverse	AACTACCTTGCCACTGACCTC
SCN2A_Ex10_F	Forward	TGAATGCCCATTTTACTCTTTG
SCN2A_Ex10_R	Reverse	GTGGAAGGCGGTTTTCTCTC
SCN2A_Ex11_F	Forward	GTCATCAGAGGGGTGCTTTC
SCN2A_Ex11_R	Reverse	GAACAAACTCCCAGCCTTTG
SCN2A_Ex12_F	Forward	GTTGTAGGAATGCTTTGGGC
SCN2A_Ex12_R	Reverse	TGAGTTATGCACGGATTTTACTG
SCN2A_Ex13_F	Forward	TTCCTCCAGCAGATTAACCC
SCN2A_Ex13_R	Reverse	TGCTAAACATTTGATGTCAAGATATAC
SCN2A_Ex14_F	Forward	AGATACTAAGTTGTTGGACCTAAACC
SCN2A_Ex14_R	Reverse	CATTTGGTGGTATGGCAATC
SCN2A_Ex15_F	Forward	GACAGTTACCTGTACATTTGCCC
SCN2A_Ex15_R	Reverse	AGACAATAGGAAGTGGCCTTG
SCN2A_Ex16_F	Forward	TGCATGTTAGAATAAAATAAGGTGTC
SCN2A_Ex16_R	Reverse	CTTAACAGGAAGGAAACACGC
SCN2A_Ex17_F	Forward	TGCATGTTAGAATAAAATAAGGTGTC
SCN2A_Ex17_R	Reverse	CTTAACAGGAAGGAAACACGC
SCN2A_Ex18_1F	Forward	TGTGACCCCTGACCTTTACC
SCN2A_Ex18_1R	Reverse	CCACAGAAAAGCCAGTTAATAATTTTC
SCN2A_Ex18_2F	Forward	GTGGGATTGATTTTCAGAAGC
SCN2A_Ex18_2R	Reverse	AGATGGTAGACAAATAAACAGACATTT
SCN2A_Ex19_F	Forward	AATAATGTAAATGAATCTCCCACC
SCN2Aex19_R	Reverse	TGGGTAATATGAGTTGATTGAAGG
SCN2Aex20_F	Forward	AACCTTCCAGCCTTAGGCAC
SCN2Aex20_R	Reverse	ACTGTGTCAGGCACCATTGC
SCN2Aex21_F	Forward	CATTGCATAGAGCAAGGCTG
SCN2Aex21_R	Reverse	TGCCAAGTATTATGGGCATC
SCN2Aex22_F	Forward	AAAATTCTTAGTTGGAGCTACCAGAG
SCN2Aex22_R	Reverse	ACATCCCTGCCAACAATG
SCN2Aex23_F	Forward	AAAACATTGCCCCAAAAGTG
SCN2Aex23_R	Reverse	ACAGAGTGATTAGAGAAAATCATCC
SCN2Aex24_F	Forward	TTCATTCCAGAGATTAACATGC
SCN2Aex24_R	Reverse	TGCATTTTGGAGGCTAAAGG
SCN2Aex25_F	Forward	ATGTGGGAGCCAATTTTCAC
SCN2Aex25_R	Reverse	TTCTAGAAATTCGGGCTGTG

SCN2Aex26_F	Forward	TTGTTGCTTTCTTAAATCAGAAG
SCN2Aex26_R	Reverse	GGGAGTTTGGGATGAATGAG
SCN2Aex27_F	Forward	GGACCTTCCAGGGTTGAGTC
SCN2Aex27_R	Reverse	GATGGGTGTTCCATCACATTC
GABRG2		
GABRG2_Ex1_F	Forward	ccacgcatgtgtaattcgt
GABRG2_Ex1_R	Reverse	aattgtaaagccgcacatcc
GABRG2_Ex2_F	Forward	cacaacctcaaggagaat
GABRG2_Ex2_R	Reverse	tccttgctctgaactacactga
GABRG2_Ex3_F	Forward	aactggtagcaaattagtgtgaat
GABRG2_Ex3_R	Reverse	ttttgctggcatgtcttttg
GABRG2_Ex4_F	Forward	ttggcaaagaaaacaggaatg
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GABRG2_Ex5_F	Forward	tgtgtttcaatcagaatgtgaga
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GABRG2_Ex6_R	Reverse	tttctgattatcattcattgagagg
GABRG2_Ex7_F	Forward	cgggcaaaaatagttttcaa
GABRG2_Ex7_R	Reverse	ttgcaggctaaatttaaagca
GABRG2_Ex8_F	Forward	gaaactgcccacagaagtca
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GABRG2_Ex9_F	Forward	tttcacttactgtgtttcaaaatg
GABRG2_Ex9_R	Reverse	tgcttaaatgtctactcataaaacaaa
GABRG2_Ex10_F	Forward	cacattggtgacattgtgga
GABRG2_Ex10_R	Reverse	GGGCTTTCACCTGTTTTATTGTC
KCNQ3		
KCNQ3_Ex1_F	Forward	gacaggctgaggctggaac
KCNQ3_Ex1_R	Reverse	gcatccatgggtcttaaagc
KCNQ3_Ex2_F	Forward	caatgtccagcagaagttgc
KCNQ3_Ex2_R	Reverse	caaccagtcaaggaggagtgc
KCNQ3_Ex3_F	Forward	accagcaacagcacaaaatg
KCNQ3_Ex3_R	Reverse	gcgtgatttccagaatagc
KCNQ3_Ex4_F	Forward	ccagcaaacagcacctatga
KCNQ3_Ex4_R	Reverse	atcaagggtggcagaatgac
KCNQ3_Ex5_F	Forward	gaccacagtcacagagctggg
KCNQ3_Ex5_R	Reverse	agctgcctggctgaacat
KCNQ3_Ex6_F	Forward	caagctgatggagaggctta

KCNQ3_Ex6_R	Reverse	aagatgacactggaaagttgagc
KCNQ3_Ex7_F	Forward	gcaattgagccctacagtcc
KCNQ3_Ex7_R	Reverse	tgcacacatgcacacataca
KCNQ3_Ex8_F	Forward	ggagtccagggcttaaacatt
KCNQ3_Ex8_R	Reverse	tgccctgaagagtgttctcc
KCNQ3_Ex9_F	Forward	gagagaagctttgcagcaatc
KCNQ3_Ex9_R	Reverse	caacagtgtgaccccaaaga
KCNQ3_Ex10_F	Forward	ccttggaggagaatgtgtgag
KCNQ3_Ex10_R	Reverse	ttaagtgggcaaaggagag
KCNQ3_Ex11_F	Forward	gcgtggaaggctttgacc
KCNQ3_Ex11_R	Reverse	ccctcccaaagtgcaagat
KCNQ3_Ex12_F	Forward	cctgccctggagtttgcta
KCNQ3_Ex12_R	Reverse	tgttggcctagacatccaca
KCNQ3_Ex13_F	Forward	attccaaacccttatctcattct
KCNQ3_Ex13_R	Reverse	cataaaggaccccagcagag
KCNQ3_Ex14_F	Forward	ggtgagaacgatggctctgt
KCNQ3_Ex14_R	Reverse	cagagtgaaccttcgtcttaaaaa
KCNQ3_Ex15_F	Forward	tttctcccaaccaatagca
KCNQ3_Ex15_R	Reverse	CACTTTGTTGTGGTGACATGG

Appendix E: PCR protocols and conditions for amplification of seven candidate genes.

PCR protocol for SCN2A

	x1
10x buffer	2 µl
dNTPs (10mM each)	0.25 µl
F-primer (5µM)	1,3 µl
R-primer (5µM)	1,3 µl
H ₂ O	13.95 µl
Taq	0.2 µl
	=19 µl
+DNA 50 ng	1 µl
	=20µl/rxn

PCR conditions for SCN2A

All exons except exon 20:			Exon 20		
	Temperature	Duration		Temperature	Duration
1	94°C	5 min	1	94°C	10 min
2	94°C	45 s	2	94°C	45 s
3	60°C	45 s	3	60°C	45 s
	-0.5°C per cycle		4	72°C	45 s
4	72°C	45 s	5	72°C	10 min
5	94°C	30 s	6	8°C	∞
6	50°C	45 s			
7	72°C	45 s			
8	72°C	10 min			
9	8°C	∞			

PCR protocol for SCN1A

x1	
10x buffer	1.5 µl
dNTPs (10mM each)	0.375 µl
F-primer (5µM)	0.5 µl
R-primer (5µM)	0.5 µl
H ₂ O	9.75 µl
Taq	0.15 µl
	=14 µl
+DNA 50 ng	1 µl
	=15µl/rxn

PCR conditions for SCN1A

All exons except exon 1:			Exon 1:		
	Temperature	Duration		Temperature	Duration
1	94°C	5 min	1	94°C	10 min
2	94°C	45 s	2	94°C	45 s
3	60°C	45 s	3	60°C	45 s
	-0.5°C per cycle		4	72°C	45 s
4	72°C	45 s	5	72°C	10 min
5	94°C	30 s	6	8°C	∞
6	50°C	45 s			
7	72°C	45 s			
8	72°C	10 min			
9	8°C	∞			

repeat 20 times

repeat 16 times

repeat 35 times

PCR protocol for SCN1B

x1	
10x buffer	1.5 µl
dNTPs (10mM each)	0.375 µl
F-primer (5µM)	0.5 µl
R-primer (5µM)	0.5 µl
H ₂ O	9.75 µl
Taq	0.15 µl
	=14 µl
+DNA 50 ng	1 µl
	=15µl/rxn

PCR conditions for SCN1B

All exons except exon 1:			Exon 1:		
	Temperature	Duration		Temperature	Duration
1	94°C	5 min	1	94°C	10 min
2	94°C	45 s	2	94°C	45 s
3	60°C	45 s	3	60°C	45 s
	-0.5°C per cycle		4	72°C	45 s
4	72°C	45 s	5	72°C	10 min
5	94°C	30 s	6	8°C	∞
6	50°C	45 s			
7	72°C	45 s			
8	72°C	10 min			
9	8°C	∞			

} repeat 20 times

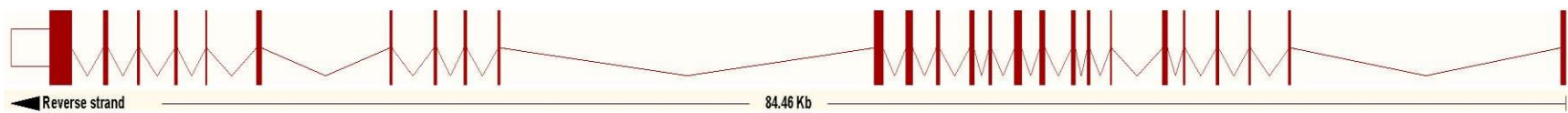
} repeat 16 times

} repeat 35 times

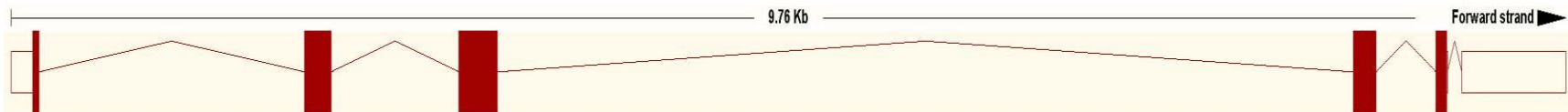
Appendix F

Candidate genes represented as exons and introns with relative sizing. Shaded blocks indicate exons and lines between blocks indicate introns. Unshaded blocks indicate untranslated regions. Strand and base pair size is also indicated on each diagram.

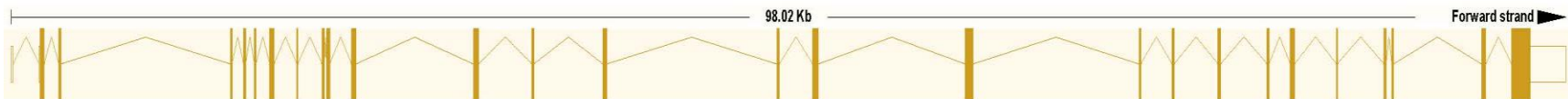
SCN1A



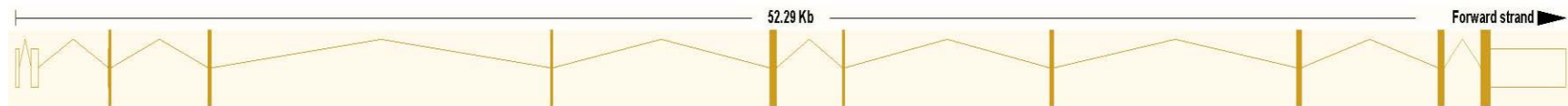
SCN1B



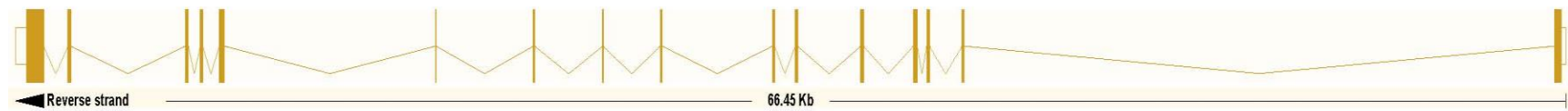
SCN2A



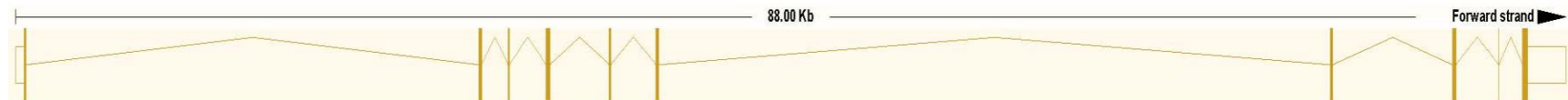
GABRA1



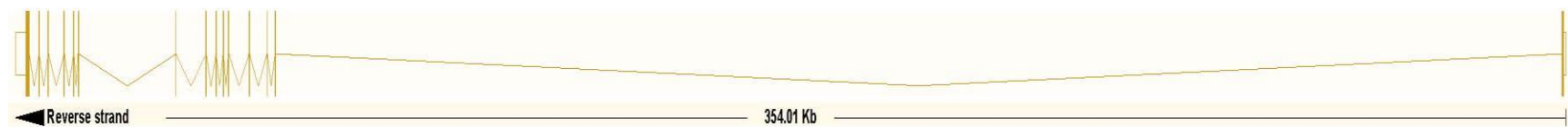
KCNQ2



GABRG2



KCNQ3



Appendix G

DNA Extraction Method, derived from:

A simple salting out procedure for extracting DNA from human nucleated cells

S.A.MWer, D.D.Dykes and H.F.Polesky

This method involves salting out of the cellular proteins by dehydration and precipitation with a saturated NaCl solution.

Buffy coats of nucleated cells obtained from anticoagulated blood (ACD or EDTA) were resuspended in 15 ml polypropylene centrifugation tubes with 3 ml of nuclei lysis buffer (10 mM Tris-HCl 400 mM NaCl and 2 mM Na₂EDTA, pH 8.2).

The cell lysates were digested overnight at 37°C with 0.2 ml of 10% SDS and 0.5 ml of a protease K solution (1 mg protease K in 1% SDS and 2 mM Na₂EDTA).

After digestion was complete, 1 ml of saturated NaCl (approximately 6M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 2500 rpm for 15 minutes.

The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to another 15 ml polypropylene tube.

Exactly 2 volumes of room temperature absolute ethanol was added and the tubes inverted several times until the DNA precipitated.

The precipitated DNA strands were removed with a plastic spatula or pipette and transferred to a 1.5 ml microcentrifuge tube containing 100-200 μ l TE buffer (10 mM Tris-HCl, 0.2 mM Na₂EDTA, pH 7.5).

The DNA was allowed to dissolve 2 hours at 37°C before quantitating.

The 260/280 ratios were consistently 1.8-2.0, demonstrating good deproteinization.